Methods to detect bacterial contamination of blood products

by

Sarah List

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This thesis is dedicated to my family, for their patience and support.

DECLARATION

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Sarah List B.Sc (Hons)
Discipline of Pathology
The University of Tasmania

ABSTRACT

Bacterial contamination of blood products presents an ongoing challenge to transfusion therapy. Contaminating bacteria are typically introduced in low numbers at the time of collection, but can proliferate during storage to reach $>10^9$ colony forming units/mL (CFU/mL). The consequences of contamination include product wastage and transfusion-transmitted sepsis, but in Australia screening is conducted on only 1% of products by a slow and labour-intensive culture method. Accordingly, there is a need for a more practicable and rapid assay. The purposes of this study were to investigate the growth kinetics of bacteria in stored products, and to develop a rapid method to detect them.

Pseudomonas, *Staphylococcus* and *Yersinia* species were inoculated into buffy coat platelet concentrates (PCs) and red cell concentrates (RCCs) at 10, 10^2 or 10^3CFU/mL and then stored at 22 ± 2°C, agitated for PCs, or at 4 ± 2°C, stationary for RCCs. Bacterial growth was monitored by plate count, and product spoilage (clumping or haemolysis) was noted. Even at the lowest inoculum, all bacterial species grew rapidly in PCs, although clumping was not observed until 3 to 5 days ($>10^7$ CFU/mL). In RCCs, all species were recovered after 36 days, but only *P. fluorescens* and *Y. enterocolitica* proliferated, reaching $>10^7$ CFU/mL before causing haemolysis.

To detect bacterial contamination, we developed a PCR-based assay. A method using bead-beating and spin column purification extracted bacterial DNA from the densely cellular blood products in under 30 minutes. Using this template and primers targeting conserved regions of the bacterial 16S rRNA gene, a 1361-bp fragment was amplified from >30 bacterial species and strains tested at less than 10^3 CFU/mL in all sample types. Examination of the PCR product using Southern blotting with specific oligonucleotide probes revealed that identification of the bacteria was also possible. Evaluation of this methodology using stored specimens from the kinetic study, showed that bacteria which had grown in blood products could be detected in 2 to 3 days

for PCs and in 6 to 9 days for RCCs. Whilst these results matched our preferred time-frame, the sensitivity of detection was reduced in comparison to directly spiked samples. This reduced sensitivity may have been a consequence of long-term storage of the test samples.

In conclusion, this study has shown that screening of blood products for bacteria using a universal PCR is feasible and practicable, and can be performed within a time frame required by the transfusion industry.

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LIST OF ABBREVIATIONS

The following abbreviations have been used throughout this thesis

A	adenine
AABB	American Association of Blood Banks
ADP	adenosine di-phosphate
AMP	adenosine mono-phosphate
ARCBS	Australian Red Cross Blood Service
ARDS	acute respiratory distress syndrome
ATP	adenosine tri-posphate
BaCon	Bacterial Contamination study
BHI	brain-heart infusion
BHI	BHI agar
bp	base pairs
C	cytosine
cc	cubic centimetres
CFU	colony forming unit
ChLIA	chemiluminescence immunoassay
CPD–A1	citrate phosphate dextrose adenine
CMV	cytomegalovirus
ddH_2O	deionised distilled water
DIG	digoxigenin-11-dUTP labelling
dNTP	nucleosidetriphosphate
DNA	deoxynucleic acid

EDTA	ethylenediamine-acetic acid
ELISA	enzyme-linked immunoassay
EVF	erythrocyte volume fraction
FDA	Food and Drug Administration
FFP	fresh frozen plasma
G	guanine
HBA	horse blood agar
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HRP	horse-radish peroxidase
HTLV	human T-cell lymphotrophic virus
kb	kilobase pair
LPS	lipololysaccharide
min	minute(s)
mL	millilitres
NAT	nucleic acid amplification test
nm	nanometres
OD	optical density
PBS	phosphate buffered saline
PC	platelet concentrate
PCR	polymerase chain reaction
PCR-ELISA	polymerase chain reaction-enzyme-linked immunoassay
pM	picamoles

PMN	polymorphonuclear lymphocytes
r	ribosomal
RBC	red blood cell
RCC	red cell concentrate
Rh	Rhesus
RNA	ribose nucleic acid
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RT	room temperature
SDS	sodiumdodecasulphate
SHOT	Serious Hazards of Transfusion study
T	thymine
TMAC	tetramethyammoniumchloride
TTBI	transfusion-transmitted bacterial infection
TTBS	transfusion-transmitted bacterial sepsis
USA	United States of America
UV	ultraviolet
vCJD	variant Creutzfeldt-Jacob Disease
vol	volume
WB	whole blood
WBC	white blood cell
wt	weight
$\times g$	times gravitational force
µL	microlitres

Awards:

1. The 2001 Australian Society for Microbiology Vic Skerman Prize. "The problem of bacterial contamination of blood products".

Presentations:

1. List SS, and SM Kirov. 2001. Bacterial contamination of stored blood products. Microbiology Australia. A127:P3.10 (presented at the Australian Society for Microbiology Annual Scientific Meeting, Perth, WA).
2. List, SS, SM Kirov, AK Bigham, and RM Robins-Browne. 2002. Rapid detection of bacteria in blood products. Microbiology Australia. PP23.2 (presented at the Australian Society for Microbiology Annual Scientific Meeting, Melbourne, VIC).
3. Addressing the challenges of bacterial contamination of blood products. Murdoch Children's Research Institute Seminar Series, 2002.
4. Addressing the challenges of bacterial contamination of blood products. University of Melbourne Department of Microbiology and Immunology Postgraduate Retreat, 2003.

Publications:

1. List SS. 2001. The challenge of bacterial blood contamination of blood products. Microbiology Australia. 22:36-40.
2. List, SS, RM Robins-Browne, and SM Kirov. Survival and proliferation of bacterial contaminants in blood components produced via the buffy coat method. Vox Sanguinis. Manuscript to be submitted November 2004.

Note: Manuscripts of the work contained in Results Chapters 4 and 5 are in preparation for submission to the Journal of Clinical Microbiology in 2005.

CHAPTER ONE

INTRODUCTION

1.1 Development of modern blood banking

The first blood transfusion was performed by a physician named Richard Lower in 1665. It followed the discovery of the circulatory system by William Harvey in 1628. Lower demonstrated with dogs that a recipient drained of its own blood had restored vitality when transfused with blood from another dog. However, 50 % of the first human to human blood transfusions conducted by James Blundell, failed. It was not until after the introduction of antiseptics by Joseph Lister in 1867 and Karl Landsteiner's Nobel Prize winning discovery of the ABO blood grouping system in 1907, that transfusion therapy advanced sufficiently for widespread application. From 1916 to 1950, many of the standard storage and typing procedures used today were developed. The first blood depots with professional donors were established in 1916. These utilised the first citrate-based anticoagulant in transfusion media. This anticoagulant prolonged blood storage and transfusion times and eliminated the need for direct suturing of donor and recipient veins for a successful transfusion. The use of Rhesus (Rh) typing in conjunction with ABO grouping eliminated a majority of transfusion reactions. The plastic bag collection system, implemented in 1950 to supersede open glass bottle systems, enabled the safe collection and preparation of multiple blood components (such as plasma and red cell products) from a single whole blood (WB) donation (67, 157, 191). Division of WB not only enabled specific treatment of blood loss or disorders, but increased the recipient to donor ratio by providing multiple products from a single donation. This is particularly beneficial with rare blood type donations where donors are scarce, as it is common for treatment to require multiple applications of one type of product. Storage of platelet concentrates (PCs) at 22 ± 2°C and red cell concentrates (RCCs) at 4 ± 2°C became standard, increasing the maximum storage duration of these products with a minimal loss of viability. Today, transfusion therapy is an important part of medicine and saves thousands of lives each year.

Thus, with most of the past transfusion problems overcome, blood services such as the Australian Red Cross Blood Service (ARCBS) now provide a range of products that are under increasing demand.

Blood banking continued essentially unchanged in theory and practice until the early 1980s, when the dramatic appearance of human immunodeficiency virus (HIV) in the blood supply demonstrated the need for continued vigilance of

donations in order to protect transfusion recipients and clinical and laboratory staff. Previously undisclosed transfusion risks such as the recent concern regarding the potential for transmission of variant Creutzfeldt-Jacob disease (vCJD, an infectious protein prion disease) continue to emerge. In light of these emerging threats to the blood supply, recent research and development has shifted toward techniques for the detection of infective blood-borne microbes in order to improve the safety of blood products and transfusion medicine. Today, viral screening of blood donations is extensive (discussed further in Section 1.4.1).

The incidence of transfusion-transmitted viral infections has declined since the introduction of screening measures, but the transmission of bacterial pathogens has remained constant (39, 103). Based on data obtained from haemovigilance programs in the USA (the Bacterial Contamination study – BaCon), the UK (the Serious Hazards Of Transfusion study - SHOT), and France, and from other fatality reports, the risk of bacterial contamination and mortality following transfusion, far outstrips the contamination and mortality rate of screened viruses (63, 83, 116, 118, 175). Haemovigilance programs have yet to be undertaken in Australia, but such reports from elsewhere show bacterial contamination is a global concern. Despite this higher risk, bacterial contamination of blood products has not attracted the same attention as viral risks. The reasons for this seeming inequity are varied and complex, but in part are due to technical limitations of bacterial screening (as discussed in Section 1.7).

Although closed blood collection systems initially reduced the frequency of bacterial contamination, developments in the preparation and storage of blood components to prolong shelf-life, potentially facilitate the proliferation of many bacterial contaminants, should these be present in the product (67, 157). Despite this, the widely accepted practice throughout the developed world is to screen only 1% of all blood products as part of a routine quality control program. Typically, screening relies on a culture method that may take several days for a result (Section 1.4.4). With no established rapid test for rigorous detection of bacteria in blood products, immunocompromised patients in particular are at risk of a severe septic reaction or death, should they receive a bacteria-contaminated product. It is clear that whilst recipients remain vulnerable to potentially fatal microbial contaminants,

the need for the development of improved rapid, specific and sensitive methods to detect bacteria in blood products is critical.

In order to address the issue of bacterial detection in blood products, it is important to clearly understand the WB collection process and the manufacture and storage of blood products themselves. This is described below, with particular emphasis on identifying the points in the collection and manufacturing process of blood products where bacteria may inadvertently be introduced.

1.2 Blood collection, product preparation and storage

The following sections detail the pre-donation questionnaire, the collection and manufacture of WB into the various blood products and their subsequent storage. These procedures, followed by the ARCBS, are based upon standards set by the Council of Europe (56).

1.2.1 Pre-donation and blood collection procedure

Prior to venepuncture at the antecubital site (phlebotomy), donors are asked a series of questions regarding their general health, their travel, history of injectable drugs, unsafe sexual practices, etc. These questions allow assessment of donor fitness and potential for carrying any unwanted transmissible agents. Donations are not accepted from donors deemed unsuitable.

The phlebotomist is required to check the donor's blood pressure and heart rate before preparing the venepuncture site with a comprehensive sterile swab of 70% isopropanol. Percutaneous phlebotomy of the antecubital vein is performed aseptically with a single-use needle conjoined to a second sheathed needle in a surrounding needle guard, which facilitates the removal of a small quantity of blood for quality control and viral screening purposes (Sections 1.3 and 1.4). The donation is then taken from the same venepuncture apparatus via a single tube connected to the collection bag.

WB donations are the most common form of blood donation, although collection of separate blood fractions (i.e. plasma, red blood cells or platelets) by apheresis also occurs. About 450 mL of WB is collected into a blood collection pack containing citrate-phosphate-dextrose anticoagulant. Different pack configurations are available depending on the type of processing and blood components to be

prepared. For example, 'top-and-bottom' packs (e.g., Optipacs, Baxter Fenwal, La Châtre, France) are used for the preparation of buffy-coat depleted products. During the donation, the blood is mixed with citrate-phosphate-dextrose-adenine (CPD-A1) anticoagulant contained in a collection bag that is cradled by a gently rocking mechanism that can detect a blood volume of 450 mL ± 10% by weight. Collection is usually completed in 10 to 20 min. If the collection time exceeds 15 min, the plasma component is considered unsuitable as a source of Factor VIII coagulation protein, and is assigned for other uses. All other components are however retained for normal processing. The bag of WB is then processed into separate components (Sections 1.2.2 and 1.2.3).

Early transfusion therapy depended solely upon WB, yet now this is not considered the most appropriate product in the treatment of a wide range of patient needs and is rarely used. Modern transfusion therapy seeks to target treatment to the clinical requirements of each recipient, using separated blood components. The major products produced by this method are RCCs, PCs, and fresh frozen plasma (FFP). This allows the direct application of the appropriate product such as RCCs in the treatment of anaemia (191). The methods for preparing these components are reviewed below.

1.2.2 Platelet-rich plasma method of preparation of blood products

The platelet-rich plasma (PRP) method is the longest used method of component preparation, and is utilised in the USA, and at some ARCBS facilities. The WB is collected and centrifuged at 640 $\times g$ to separate the plasma and red blood cells (RBCs), the latter of which is expressed into a new component bag as a finished RCC. Final PRP RCCs contain a maximum of 0.2 to 1 $\times 10^9$ (ARCBS standards) white blood cells (WBC)/unit. The platelet-rich plasma is further processed at 1000 $\times g$ to separate the cellular platelet portion from the plasma. The final PRP PC contains 0.05 to 1 $\times 10^9$ WBCs per single unit equivalent (they can be pooled together) (56).

1.2.3 Buffy coat method of preparation of blood products

The buffy coat method is widely used for blood product preparation in Australia, and also is used extensively throughout Europe for reasons detailed below. Separation of blood components by the buffy coat method is depicted in Figure 1.1. The WB is collected into 'top and bottom' packs and is centrifuged at 5 000 $\times g$ for 10 min, and the RCCs and FFP are expressed into separate satellite bags using an automated blood component separator (e.g., Optipress, Baxter) leaving the platelet-rich buffy coat in the primary pack. Buffy coats contain 50 to 70% of leukocytes from the initial WB donation, and a small percentage of RBCs in addition to the platelet fraction (56). The RBCs are resuspended in 100 mL storage solution (e.g., Adsol solution, Baxter Fenwal, Deerfield) making a finished RCC. PCs are prepared by pooling four or five ABO and Rh compatible buffy coats into a platelet bag (e.g., PL2410 bag, Baxter). These are then resuspended in 300 mL plasma or platelet additive solution (e.g., T-sol®, Baxter Fenwal), and re-centrifuged at 2 000 $\times g$ for 2 to 3 min. The platelet-rich portion of the buffy coat is siphoned into a fresh bag, and the remaining leukocyte-rich fraction is discarded. The final PCs contain approximately 8×10^7 WBC/unit, whilst RCCs contain around 10^8 WBC/unit (56). The advantages of the buffy coat method over other WB separation procedures such as the PRP method, include a reduction in leukocyte contamination and red cell aggregation and an increased recovered platelet yield from the buffy coat (56). The standard blood products can be further processed into what are known as 'boutique' blood components (e.g., passed through a WBC-removal filter to deplete these to less than 10^6 WBC/unit) which are usually manufactured upon request.

This thesis shall focus on the standard cellular blood products produced by the buffy coat method (i.e., PCs and RCCs) as these are most risk of high levels of contamination, they are most commonly used in Australia, and little is known about how bacteria behave in these during storage. However, it is anticipated that detection methods produced by this work shall be applicable to all blood product types.

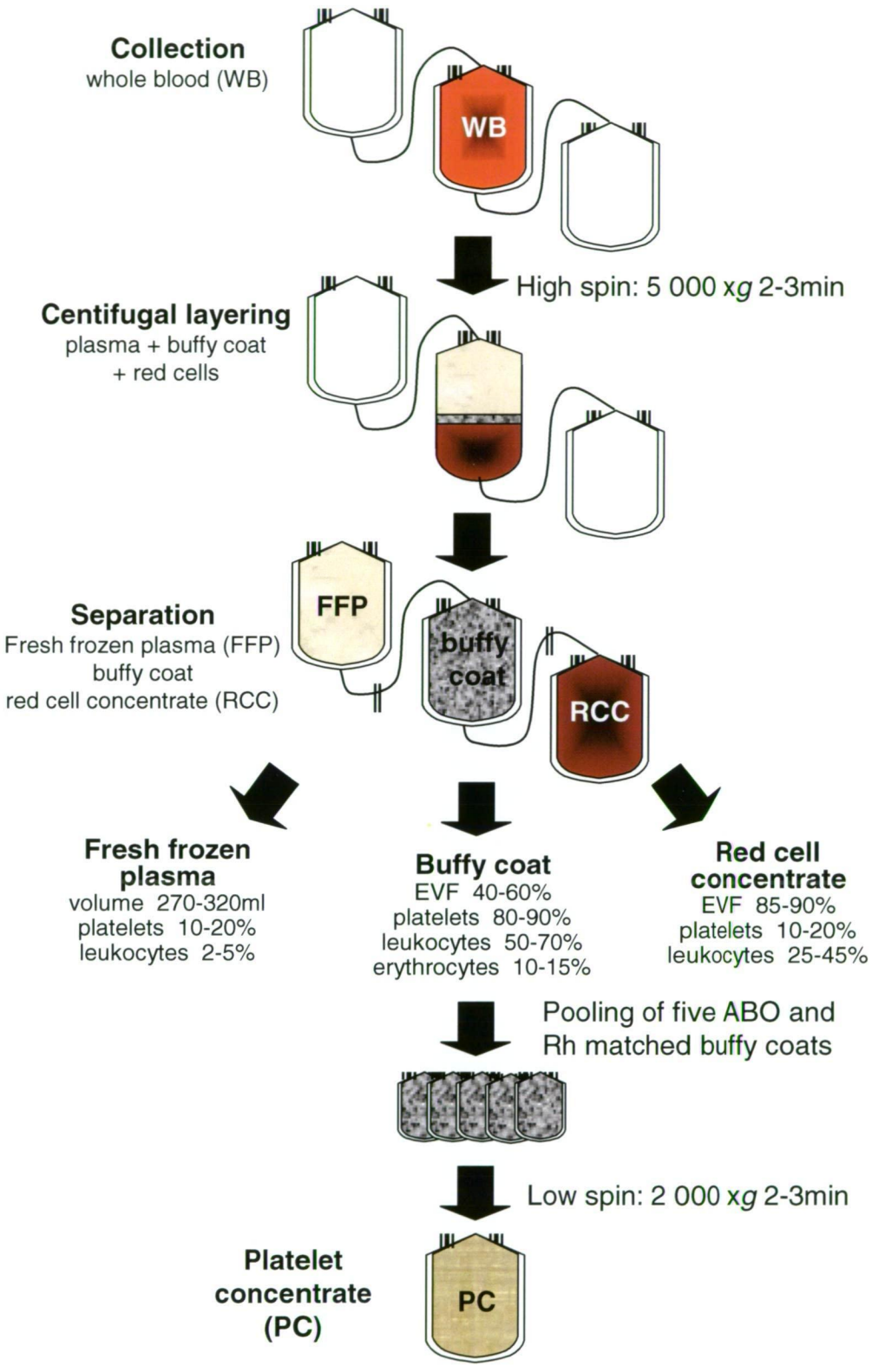


Figure 1.1: Diagrammatic representation of the method used to derive buffy coat blood products

EVF – Erythrocyte or cellular volume fraction

1.2.4 Storage of blood products

Separating WB into products by the buffy coat method, results in products with distinctly different cellular contents and hence, potential applications. Component preparation also facilitates optimal product storage for the viability of the unique cellular content of each. Blood collection and storage uses anticoagulants that contain buffered citrate to prevent coagulation, and dextrose (glucose) as an energy source to maintain cell viability and extend the shelf-life of the product (56). Other additives in anticoagulants and storage solutions include adenine and phosphate, as will be detailed in the sections following.

1.2.4.1 Fresh frozen plasma (FFP)

FFP is frozen under conditions that will maintain its labile coagulation factors in a functional state (56). As FFP is stored at sub-zero temperatures (usually -25°C or less), the concerns related to FFP are contamination with cellular and viral matter rather than transfusion-transmitted bacterial sepsis (TTBS). TTBS following transfusion of FFP is a rare occurrence in comparison to products stored in the liquid state, notably PCs and RCCs.

1.2.4.2 Red cell concentrates

Storage at 4 ± 2°C in Adsol storage solution permits up to 42 days of RCC viability. There are, however, a number of considerations that arise by this length of storage. The acidity increases over time, reducing glycolysis and the content of adenosine nucleotides (ATP, adenosine di-phosphate [ADP], and adenosine mono-phosphate [AMP]), essential for cell membrane and transport functions. The latter has been addressed by the addition of adenine to the storage solution, which as the main component of the adenosine nucleotides allows the RBC to synthesise new nucleotides to compensate for the losses incurred. Aggregate formation by platelets and leukocytes (microaggregates), require product filtering at the point of transfusion and the oxygen-carrying capacity of the red blood cells decreases (56). The duration and temperature of RCC storage, and their high iron content provide suitable conditions for the proliferation of psychrophilic bacteria (organisms capable of growing at less than 4°C)(200). This concern is considered in greater detail in Section 1.5.2.

1.2.4.3 Platelet concentrates

PCs, such as those produced by the buffy coat method present the greatest challenge of modern blood collection, as they require storage at 22 ± 2°C and continuous oxygenation to ensure component viability and longevity. Furthermore, the pooling of buffy coats increases the chances of contamination of the final PC.

PCs are also manufactured by apheresis, which allows collection of one platelet donation from a single donor. It involves the separation and retention of the platelet component from WB whilst the remainder of the blood is returned to the donor. All apparatus that is in direct contact with the donor blood during the collection is for single use only and is sterile and disposable. This eliminates the possibility of donor and product cross-infection via the apheresis machinery. The pre-donation questionnaire and the selection of platelet donors are identical to those used for WB donors. Apheresis requires a single donation over a one hour period. It produces number of platelets equivalent to PCs such as those produced by the pooled buffy coat method. Despite the improved platelet yield and the purported reduction in the frequency of bacterial contamination (138, 145, 146, 225), apheresis PCs are not produced as frequently as buffy coat PCs. This is due to the considerably higher expense involved in apheresis collection, the longer time required for the donation, and the limitation that just one product is obtained by this method as opposed to the three by the standard buffy coat method. Storage of apheresis single donor and pooled buffy coat PCs is identical (56). For the purposes of this thesis, the abbreviation PC will simply refer to both apheresis and buffy coat methods of production of PCs.

Under the described storage conditions, platelets maintain viability and function for seven days, but storage is currently limited to five days (132). This is due to safety concerns, because if bacterial contaminants are present there is a significant risk of high bacterial load after seven days of storage. Bacterial contaminants were most commonly found in significant numbers after five days of storage, causing a significant number of episodes of TTBS (34). Consequently, in 1986 the Food and Drug Administration (FDA) in the USA limited the storage of PCs to a maximum of five days in order to reduce the incidence of PC-related TTBS (6). As the main focus of this thesis, the problem of bacterial contamination in the context of PCs and RCCs will be described in greater detail in Section 1.5.2.

1.3 *Product quality control*

Quality control of product content is conducted at the ARCBS on two levels: mandatory screening of blood donors and representative portion screening of manufactured blood products. Mandatory screening is predominantly conducted immediately following the collection process, and includes ABO grouping, haemoglobin content, and targeted viral screening (detailed in Section 1.4). Approximately 1% of each product type is screened for other qualities, such as Factor VIII content in FFP (Table 1.1).

1.4 *Microbial screening*

As mentioned in Section 1.1, the screening of blood donations has been developed and implemented for the protection of the recipient and staff, and to ensure the integrity of the blood supply. The screening methodology for each category of contaminant varies depending on the nature and physical structure of the contaminant concerned, and the relative ease, speed, specificity and sensitivity with which each may be detected. Extensive testing methodologies are now available for viral contaminants. However, the methodologies for detecting bacterial and protozoan contaminants have been less rigorously developed.
The major focus of this thesis is the bacterial contamination of blood products. Hence, although it will be discussed briefly here (screening for Syphilis and generic bacterial screening), it will be covered in greater depth in Section 1.5 onwards.

The following Sections will also review the viral and protozoan contaminants of greatest concern to transfusion medicine, how they are introduced into products, and the techniques in place to detect them and prevent their transmission to the recipient.

Table 1.1: Quality controlled characteristics of blood products

(Adapted from Contreras, 1998)

Mandatory screening (WB)	**1% Screening (blood products)**
ABO grouping	*RCCs:* volume, residual WBCs, residual platelets and packed cell volume
Rh D typing	*PCs:* volume, platelet count, residual WBCs, residual RBCs and pH
RhC and E typing	*FFP:* volume, factor VIIIC content
Antibodies to red cell antigens	Cryoprecipitate: volume, factor VII content
Haemoglobin	
HLA typing (when required)	

1.4.1 Viruses

Viruses are obligate intracellular parasites that are responsible for numerous life-threatening diseases. Blood-borne viruses such as hepatitis C virus (HCV) can be highly infectious, with high mortality rates. Hence, screening for virus infection constitutes the most extensive branch of testing performed on blood products. Detection at the ARCBS is achieved by two methods: serological-based assays, such as the currently used chemiluminescence immunoassay (ChLIA – Abbott PRISM), and nucleic acid amplification test (NAT – Chiron Gen-Probe). Both assay systems are specifically designed for high-throughput screening. Table 1.2 shows the viruses screened and the methods by which they are detected at the ARCBS.

Serological assays for viral antigen/antibody detection have been in routine use for many years. The automated Abbott PRISM ChLIA-based assay system is a more recent development. The virus-serological assays are sensitive and specific, but are dependent upon the seroconversion status of the individual. That is, donors that are infected and producing antibodies in response. If the donor has not yet seroconverted (as happens during the 'window period'), a false-negative result may be obtained. HIV infection produces a 22 day window period, while HCV may have a window period of up to 70 days (2, 59, 101, 131). A further limitation is that sensitivity and specificity of the test can be compromised, such as when samples are frozen prior to testing.

To overcome these issues, the ARCBS also employs the NAT, which currently detects HIV and HCV. Detection of other viruses by the automated Chiron NAT system is currently being developed.

With this system, the viral nucleic acid (if present) is amplified and detected by hybridisation protection assay utilising a chemiluminescence-coupled complementary RNA probe that is quantitated (in relative light units) in a luminometer (8). The NAT technique reduces the window period by up to 14 days for HIV and by up to 30 days for HCV (2, 93). While the NAT technique is not fail-safe, it offers increased assurance of blood product safety.

Table 1.2: Mandatory viral screening tests currently applied to all blood donations in Australia

(Kiely, 2004)

Virus	Serological screening (CHLIA)	Nucleic acid Amplification testing (NAT)
HIV-1 and 2	Yes	Yes
HBV	Yes	No
HCV	Yes	Yes
HTLV-1 and 2	Yes	No
CMV	Yes (as required)	No

1.4.2 Parasites

In Australia, transmission of parasites by blood donation is rare, as any individual who may have been exposed to parasitic infections by clinical or travel avenues within the preceding 6 months, will be excluded at the pre-donation questionnaire stage (56).

Malaria is the most common parasitic disease of concern. It generally occurs between the latitudes 45 degrees north and 40 degrees south of the equator, although cases have been reported elsewhere. The causative agents of malaria are *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *P. falciparum* is the most dangerous of these human parasites as it can infect the brain (75). Natural transmission occurs by the *Anopheles* mosquito, followed by a 12 day to 10 month incubation period. The malarial parasite is restricted to within RBCs, which may then contaminate blood products containing either residual (e.g. PCs) or many (e.g. RCCs) RBCs. Invasion and multiplication within the RBC eventually leads to the destruction of the host cell, the release of new progeny, and anaemia (95). Clinical symptoms of infection reappear with each round of parasitic multiplication. Travellers who have been in an area considered endemic for malaria are excluded from donating for one year following travel, whilst immigrants from such areas are precluded for a period of 3 years (56). Each of the above parasites can survive storage at 4°C for more than a week.

1.4.3 Syphilis (*Treponema pallidum*)

The bacterial spirochete, *T. pallidum* is normally transmitted sexually, but can also be transmitted congenitally and by fresh blood and PCs. The sexually-transmitted infection progresses through three stages: the primary, characterised by a lesion at the site of infection known as a 'chancre'; the secondary, whereby the bacteria become blood-borne; and the tertiary, where the bacteria leave the blood stream and invade the heart, musculoskeletal system and central nervous system. Symptoms such as a rash and fever may be absent from the infected individual at the primary and secondary phases, and the symptoms of tertiary syphilis including heart damage, neurological symptoms and skin lesions develop over a period of years or

decades. Treatment requires antibiotics and is most successful if the disease is diagnosed at an early stage of infection. Screening for *T. pallidum* is by a serological assay which detects anti-cardiolipin antibodies. Cardiolipin is a lipid normally found in mammalian mitochondrial membranes and hence, it is not normally in contact with the immune system. The lesions associated with syphilis presumably are responsible for the release of cardiolipin, although viral infection, autoimmune disease and some malignancies can generate the same lipid release and result in false-positive detection (58, 171). Also, in early primary syphilis at the height of infectivity, screening tests may be negative. Although it has been reported that *T. pallidum* is inactivated by refrigeration for >72 hours (53), mandatory precautionary screening occurs at the ARCBS.

1.4.4 General bacterial contaminant screening

In Australia, the approach used to detect bacterial contaminants in samples of blood products utilises automated culture, such as BacTAlert (Organin Teknika, now Bio Mérieux) whereby positive samples are identified by increasing levels of CO_2 within the cultures bottles as the bacteria proliferate. As this process is dependent on the rate of proliferation of the bacteria, this method may take up to seven days for a positive result to be recorded. Automated culture may also be subject to false-positive results (i.e. reculture is negative following BacTAlert positive detection). In countries such as Belgium, it has been reported that more than a third of initially positive automated cultures are negative upon reculture (183). Some countries also employ a Gram or acridine orange stain for all blood products prior to transfusion. However, this is not the case in Australia as these tests are not considered sufficiently sensitive for accurate detection of bacterial contaminants at lower concentrations (discussed in more detail in Section 1.7.3) (86).

Following the positive identification of a contaminated product, the bacterium is identified by a commercial API kit (Bio Mérieux). This consists of a strip of colour reaction tests investigating the fermentative abilities of different bacteria. Cultures are grown on a range of semi-solid media (chocolate, MacConkey and blood agar) under aerobic and anaerobic conditions for 24 to 48 hours and are Gram-stained. The results of these preliminary tests and the identified morphology of the bacterium (rod, coccus) enable selection of an appropriate API test for the

suspected organism. The API test results are then compared with the standardised test results of various organisms.

1.4.4.1 Common bacterial contaminants

The bacterial contaminants commonly isolated from blood components in the USA and Australia are shown in Figures 1.2 and 1.3. There appears to be a considerable difference between the organisms isolated in each country (e.g., more skin commensals are evident from the Australian data). However, it may be due to the variation in donor populations between these countries, as until recently, most blood banks in the USA were paid operations, whilst the ARCBS donors are voluntary. It is widely believed that voluntary donation systems attract 'healthier', (hence lower risk) donors. Also, the Australian data for the bacterial species identified in blood products have been obtained by the screening of 1% of all blood products manufactured, whilst the results from the USA were obtained from culture of the remaining product after a reported episode of suspected TTBS (116, 175). It could, therefore, be concluded that the Australian data provides a profile of the complete spectrum of bacteria that contaminate and persist in blood products, while the data from the USA represents the organisms most frequently causing sepsis following inadvertent transfusion of bacteria-contaminated products.
This suggestion is supported by the similarity of microorganisms obtained from prospective surveys conducted elsewhere, to those found by the Australia survey (14, 22, 25, 65, 119, 128, 174, 225). The combined data give a diverse array of more than 35 bacterial species, and these can be introduced into the products in a number of ways.

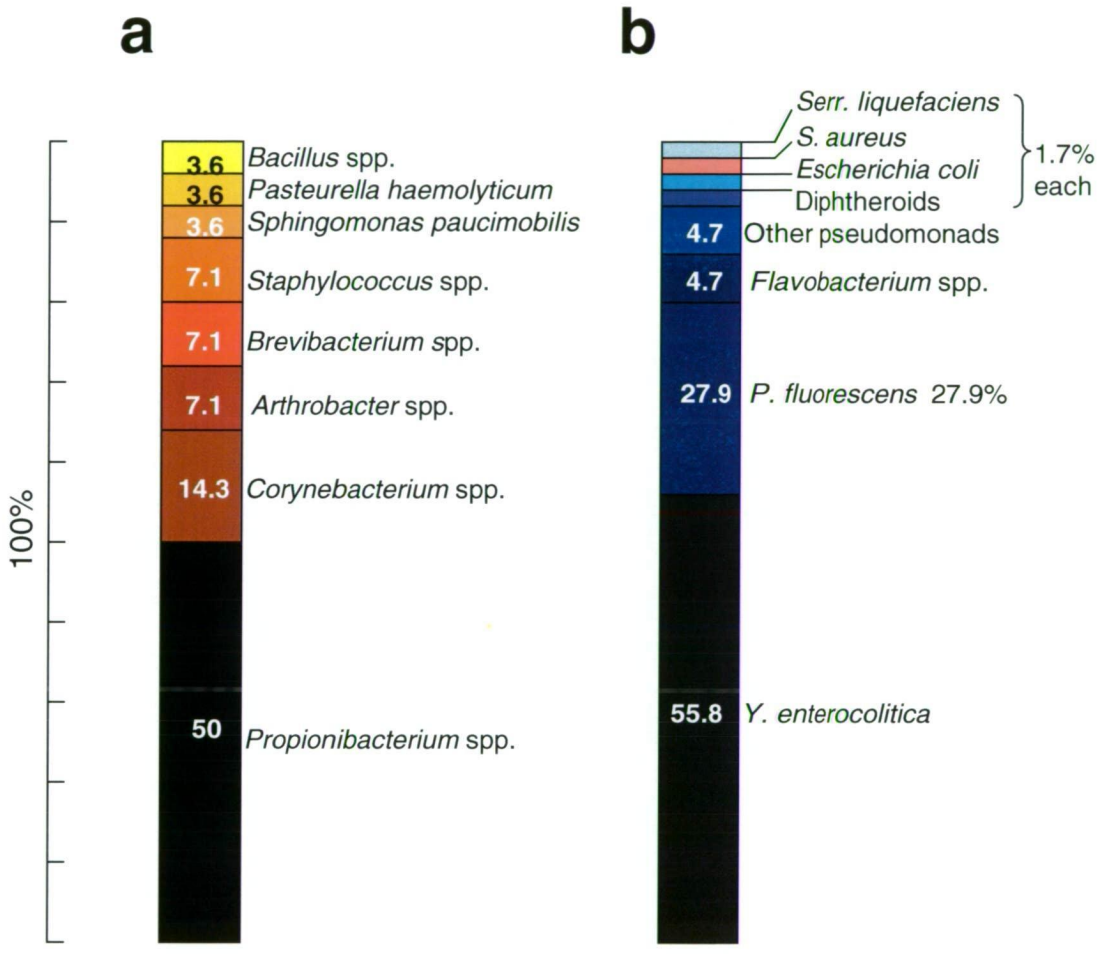


Figure 1.2: Bacteria in red cell concentrates

a) Common contaminants found in 1% of RCCs by routine automated culture.

b) Species indicated in RCC transfusion-transmitted bacterial sepsis. (data from Kuehnert, 2001; Roth, 2001; and Baleriola-Lucas, 2001)

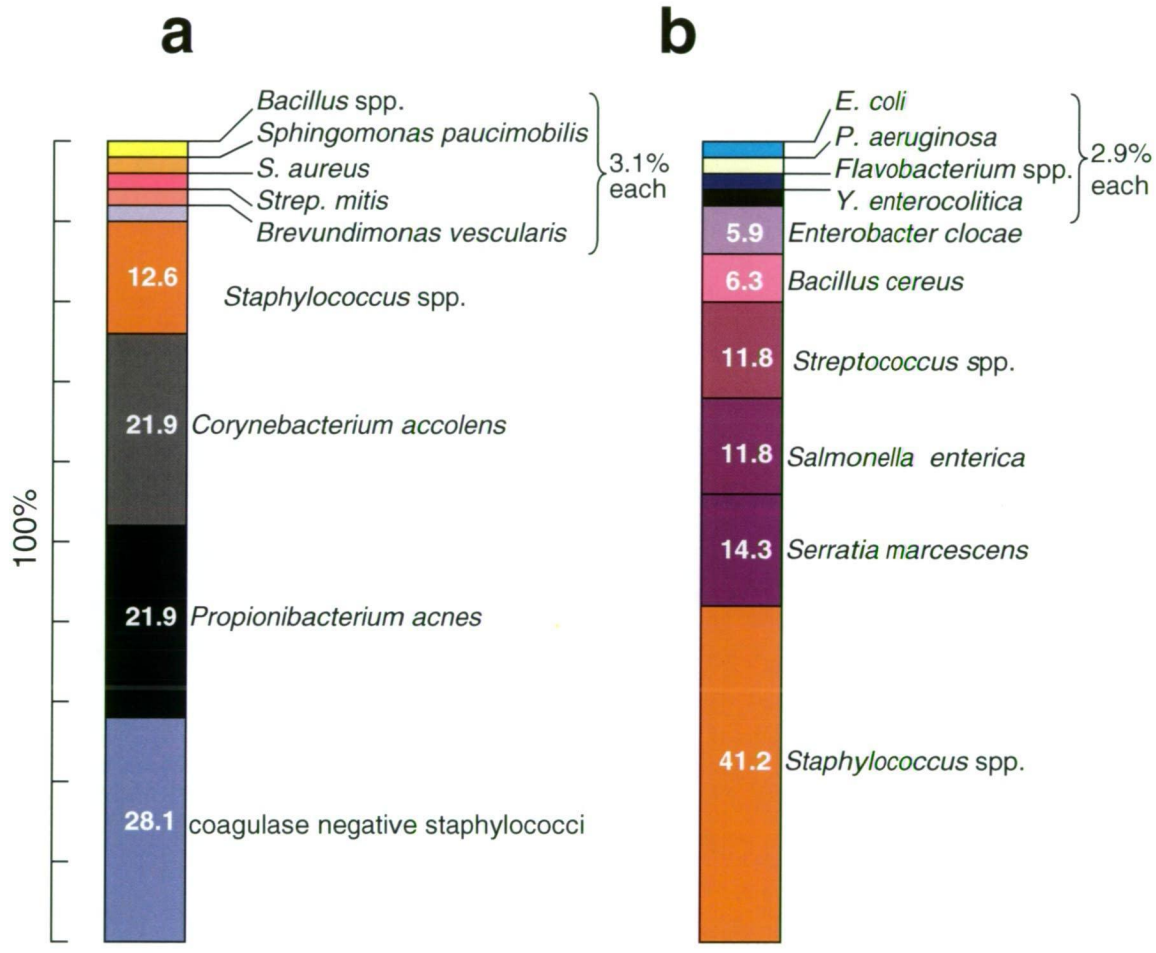


Figure 1.3: Bacteria in platelet concentrates

a) Common contaminants found in 1% of PCs by routine automated culture.

b) Species indicated in PC transfusion-transmitted bacterial sepsis. (data from Kuehnert, 2001; Roth, 2001; and Baleriola-Lucas, 2001)

1.5 Bacterial entry and establishment in blood products

The contamination of blood products by bacteria can occur by several pathways. Bacteria are usually introduced at low numbers (5 to 10 CFU/mL) into the WB or blood products (7, 24, 57, 76, 78, 201). However, long periods of storage under conditions that allow bacterial proliferation, provide opportunity for the contaminants to multiply to clinically significant numbers (>10^5 CFU/mL) (226) and pose a risk to recipients. However, entry does not guarantee the survival or proliferation of the organism. These issues are addressed below.

1.5.1 Routes of contaminant entry

Contamination occurs most frequently through incomplete antisepsis of the venepuncture site, but may also occur following donor bacteraemia, or processing with non-sterile equipment.

1.5.1.1 Incomplete skin disinfection

There is considerable evidence that suggests incomplete disinfection at the antecubital fossa is a common occurrence. The most frequently implicated microorganisms in TTBS are skin flora such as *Staphylococcus epidermidis* (hereafter referred to as *S. epidermidis*) and *Staphylococcus aureus* (*S. aureus*). Moreover, Goldman (80) showed that antiseptic skin preparations are not always effective. In that study, volunteers were subjected to disinfection of the antecubital site with a range of antiseptics on discrete occasions. Quantitation of the contaminants post-disinfection was by contact plate count, and each contaminant was identified. The most effective method appeared to begin with a 70% isopropanol scrub followed by a 2% tincture of iodine scrub. However, the findings of this study are limited by the fact that no contact plate counts of the antecubital fossa bacterial load pre-disinfection were taken for comparison with the post-disinfection count. An earlier study reported a 2% chlorhexidine preparation was more effective than either a 10% povidone-iodine or 70% alcohol swab in skin antisepsis, and also provided a residual antibacterial activity for several hours after application (125). The ARCBS currently utilises a simple 70% isopropanol scrub for disinfection, but in light of the above findings, this is under review.

A further complexity to the skin antisepsis problem as reported by Anderson (5), was that despite careful iodophor disinfection, `dimpling' of the antecubital fossa in long-term donors led to a culture of coagulase-negative staphylococci in repeat blood samples taken from the area. Blood taken from non-dimpled sites was culture-negative. This may be due to a small core of skin entering the collection bag at the time of donation through the venepuncture needle, as WB contamination persisted despite the fact that post-disinfection surface sampling of the antecubital site yielded no bacterial growth (77, 79). This suggests that despite an effective and complete surface disinfection of the venepuncture site, surface disinfection alone is insufficient to prevent the contamination of WB donations. The ARCBS has attempted to reduce the possibility of contamination in this way by avoiding the venepuncture of dimpled skin in all donors, although there has been no investigation into the efficacy of this practice in a large-scale operation such as a blood bank.

1.5.1.2 Donor bacteraemia

Although most people who are bacteraemic are also symptomatic, episodic bacteraemia may occur in people who appear well (79). The lack of visible symptoms in a healthy individual may be due to a number of reasons, such as a low concentration of microorganisms in the blood during the incubation or recovery period of infection, or during a long-term asymptomatic carriage of the bacterium (79). When combined, these present particular difficulty in assessing the health of a potential donor, and are likely to be responsible for a substantial number of all bacteria-contaminated products. The organisms most commonly implicated in the contamination of blood products via this route are *Y. enterocolitica* or *Salmonella enterica.* which can invade the intestinal mucosa and become blood-borne (57, 90).

Epidemiological evidence largely supports this idea, as one third of WB donors implicated in *Y. enterocolitica* transmission via red cells products, have been reported to have experienced gastrointestinal illness (prior to, during or soon after donating blood) which is an established hallmark of *Y. enterocolitica* infection. *T. pallidum* is also a blood-borne pathogen that can mediate TTBS, although this rarely occurs. Contaminants acquired via this route have also been detected in other blood components such as PCs and FFP, and caused infection following transfusion of these products. A notable, yet unusual outbreak of *Salmonella* Cholerae-suis

followed the apheresis extraction of plasma from an asymptomatic donor. It was later inferred that the patient unknowingly had osteomyelitis of the tibia with low-grade chronic bacteraemia (169). Dental procedures and even toothbrushing have occasionally been implicated as the source of organisms such as *S. aureus*, *Serratia liquefaciens* (hereafter referred to as *Serr. liquefaciens*) and viridans group streptococci (19, 31, 79, 147, 154). The bacteraemic phenomenon, however, appears transient when donor infection occurs via this route, and potential blood donors who have undergone any dental work are only deferred from donating blood on the same day to prevent possible transmission of infection as a result of such procedures (82, 147, 200).

1.5.1.3 Non-sterile equipment

Contaminated equipment has been linked to several episodes of TTBS although this route of contamination is now rare since the development of closed blood collection systems. The most recent report of such contamination involved a simultaneous outbreak of *Serr. marcescens* in Denmark and Sweden. This was subsequently traced to a batch of contaminated single-use blood collection containers from which a ribotype analysis revealed that the organism from the contaminated products matched an isolate obtained at the manufacturing plant (91, 99).

1.5.1.4 Contamination during processing

AuBuchon (10), simply yet elegantly demonstrated that the use of an aseptic blood-bag side tube sealing device does not necessarily guarantee a sterile product. When 244 seals made by the device were challenged externally to the seal with bacteria, pack sampling resulted in two being positive for contamination, indicating bacteria could enter through what was considered to be an aseptically produced seal. It was subsequently recommended that all welds be visually inspected when produced, as both in this case were found to be incomplete. If contaminated, 37°C waterbaths provide ideal conditions for the growth of many bacterial species, and it is reported that up to 54% of all episodes of product contamination following thawing of FFP have resulted from the introduction of bacteria from a waterbath to the product through microscopic cracks in the container (212). This risk, can however, be avoided by the use of sterile container 'overwraps' in conjunction with

regular disinfection of the waterbath, or by use of the alternative of microwave thawing of frozen products (44, 212).

1.5.2 Factors influencing bacterial establishment in blood products

As mentioned previously, the introduction of bacteria into WB or blood products does not guarantee their survival or proliferation. Some bacteria have fastidious nutritional or conditional growth requirements which may not be met by the product, or it may be out-competed for the limited resources by a more quickly adaptive and opportunistic co-contaminant. Moreover, as an intrinsic part of the complex human immune system, blood is also capable of generating a natural antimicrobial activity that may reduce or eliminate the microorganism prior its establishment (32).

1.5.2.1 Blood component characteristics and storage

The storage temperature and properties of each blood product have been chosen to optimise the preservation of the products during storage. However, the temperatures used also act to restrict the range of contaminants to organisms capable of inhabiting and multiplying in those specific niche environments. This will now be explored further for red cell and platelet products. FFP and other products that are stored in the frozen state will not be considered further as they have only on rare occasions been implicated in TTBS.

The storage of RCCs under refrigeration (4 ± 2°C), whilst allowing the survival and persistence of many species of bacteria, restricts proliferation to species which are psychrophilic. Most bacteria studied previously in PRP products, which are able to proliferate under these conditions, experience a growth lag period of at least 7 days in RCCs, followed by a gradual exponential increase in concentration over the next 14 days (7, 73). The psychrophilic pseudomonads aptly fill these criteria. However, there are some organisms, not strictly considered psychrophilic that successfully proliferate in RCCs. *Y. enterocolitica* is not considered psychrophilic, yet in the USA it is responsible for more than 50% of all contaminated red cell products tested and is able to proliferate at 4°C (34). Growth of this organism may be enhanced by the long storage period of RCCs, and the plentiful availability of iron. Bacterial species including *S. aureus* and *S. epidermidis* which

are also not considered psychrophilic have also been reportedly linked to episodes of RCC patient sepsis, and this may be due to the production of bacterial superantigens or other toxins (116, 164, 216, 217). Further information about the behaviour of these bacteria in stored blood products would therefore be highly useful in explaining the mechanisms of patient disease in these cases.

A larger and more diverse range of contaminants have been found in PCs (especially pooled buffy coat PCs) than RCCs, due to their storage at 22 ± 2°C with constant agitation (Section 1.2.4.3). They are generally considered as the product of greatest infectious concern to transfusion medicine (23). Most organisms isolated from PCs are skin saprophytes. They include *S. epidermidis* and *Propionibacterium acnes* (hereafter referred to as *Pr. acnes*), or perpetrators of gastroenteritis such as *Salmonella enterica*, *Bacillus* spp., or *E. coli*. Notably, many of these organisms are aerobic, thriving in the high oxygen tension of PCs. Anaerobes also multiply freely in PCs, provided they are not obligate anaerobes. Typically, PC contaminants spiked into PRP PCs exhibit a lag phase of 24 hours, followed by an exponential growth of up to approximately 10^8 CFU/mL (160, 201). The concern over the contamination of PCs with bacteria, limits storage to 5 days although viability of the product can be maintained for 7 days (Section 1.2.4.3). Although this practice reduces the risk of transfusion of products containing significant numbers of bacteria, it leads to significant challenges for efficient product inventory management.

1.5.3 Prevention of contamination

Despite the apparent ease with which bacteria can enter blood products, there are a number of procedures that may assist in the prevention of this.

1.5.3.1 Blood diversion

Several studies have shown that the first 10 to 15 mL of WB taken, are more likely to contain bacteria than further samples (36, 147). Bruneau (36) recorded a 72% reduction in the number of contaminated products if this precaution was taken, confirming the finding that contamination occurs most frequently at the time of collection (189). However, 100% sterility of products remains unobtainable, and this procedure is not in use at the ARCBS.

1.5.3.2 Whole blood holding period

Freshly-drawn blood contains WBCs which phagocytose bacteria, and plasma which contains complement proteins to suppress bacterial proliferation (6). This has been exploited by the ARCBS, where an overnight WB holding period following collection is in place. Evidence from Högman (100) also supports that WBC have a bactericidal effect upon a range of organisms including *S. epidermidis*, *S. aureus*, *Pseudomonas aeruginosa* (hereafter referred to as *P. aeruginosa*) and *Propionibacterium* spp. following inoculation into WB of between 1 to 100 CFU/mL. Within 5 hours, all organisms tested were reduced by at least 1 to 2 logs in WB containing WBCs, in comparison to WBC-depleted WB. It has not, however, been investigated whether this phenomenon is dependent upon the WBC count. That is, whether a higher concentration of WBC achieves greater bactericidal effect than a lower concentration (although this would be a logical hypothesis). There is also some debate into the legitimacy of the Högman (99) study as the bacterial species were cultured *in vitro* and were not grown in blood. As many virulence factors are located extrachromosomally on plasmids that may be 'lost' when bacteria are cultured, this may render the bacteria particularly vulnerable to phagocytic killing by WBC (such as happens with *Y. enterocolitica* when kept at 22°C) (98, 148). Therefore repeated studies should be conducted by selectively culturing the bacteria to retain their virulence plasmids, to determine if the results observed were real. Furthermore, some investigators have suggested that WBCs should be filtered from the products following the holding period, as evidence suggests that some bacteria (such as *Y. enterocolitica*) may remain viable following phagocytosis, and escape upon the death of the phagocyte (66, 100).

Should bacteria be able to enter, establish and proliferate, spoilage of these blood products is inevitable. More serious are the risks posed to recipients of such contaminated products.

1.6 Transfusion-transmitted bacterial infections

The symptoms of bacterial septic reactions (listed in Table 1.3) are relatively non-specific, making recognition a complex problem, particularly with the variety of clinical sequelae that may occur post-transfusion. There is some debate as to whether particular clinical manifestations can be used as a direct guide to the

diagnosis of TTBI, as most symptoms are identical to those of other acute haemolytic transfusion reactions, and further may lead to misdiagnosis (86). Symptoms of TTBI usually present within the first few hours, with more than 70% of TTBI cases first presenting symptoms during the transfusion (86). Recipients have reported a violent chill during or immediately following the application of the contaminated product.

This may be accompanied by an increase in polymorphonuclear neutrophils (PMNs) in the blood, fever, tachycardia, hypotension, nausea, vomiting and circulatory collapse in the case of progressed septic shock (28, 141, 142). The outcome of septic shock varies depending on the recipient's immune system, its early recognition, the immediate discontinuation of the transfusion, and the application of appropriate antibiotics and additional supportive measures (6, 79). Atypical symptomatic TTBI cases are rare, but it is important to attempt to identify the most common sequelae, as if recognised and treated early, more than 80% of infected individuals survive. More than 70% die if the infection progresses to septic shock.

1.6.1 Bacteraemia

The term 'bacteraemia' refers simply to the presence of bacteria in the blood, which although a clear indication of infection, may not manifest in visible symptoms (6). This is especially true in healthy individuals where contaminating organisms are quickly removed by immune mechanisms such as phagocytic cells in the blood, or the Kuppfer cells in the liver (6). Unlike healthy donors, the state of bacteraemia in immunocompromised transfusion recipients can quickly lead to established disease such as sepsis.

The bacteraemic condition as a precursor to sepsis is exceptionally concerning as all patients transfused with bacterially-contaminated products could be considered bacteraemic and are hence at a high risk of sepsis, particularly as most recipients are immunocompromised.

1.6.2 Sepsis, septicaemia, and septic shock

Sepsis is a condition that results from bacteraemia, and involves the putrefactive destruction of tissues by disease-causing bacteria or their toxins, accompanied by a febrile reaction (209, 213).

Table 1.3: Frequency and type of transfusion-transmitted bacterial infection symptoms

(Adapted from Greene, 1995)

Symptom	Frequency (%of TTBI cases)
Fever	79
Chills/Rigors	74
Hypotension	70
Disseminated intravascular coagulation	33
Nausea	30
Vomiting	28
Acute respiratory failure	26
Acute respiratory distress syndrome	16
Diarrhoea	16
Tachycardia	16
Back pain	16
Intravenous site pain	12
Urticaria	5
Rhabdomyolysis	2
Abdominal pain	2

Sepsis leads to 'septicaemia', a systemic disease in which the organism continuously multiplies in the blood. The symptoms of sepsis are the same for both Gram-positive and Gram-negative organisms. Septic shock is triggered when bacterial cell wall components or lipopolysaccharide (LPS) are released into the blood, although the mechanisms for the sequelae of events remain poorly defined (151). Following bacterial lysis, it is thought that LPS is bound by an LPS-binding protein, which in turn binds the CD14 molecule on monocytes and macrophages (Fig. 1.4). The LPS-LPS binding protein complex also has the ability to bind to surface receptors of epithelial cells. Following complex binding with the host cells, cytokines such as IL-1, IL-6, IL-8, TNFα and platelet activating factor (PAF) are produced which in turn stimulate a range of physiological responses. Amongst other functions, IL-1 causes an increase in PMN numbers and stimulates prostaglandin production, causing fever. When injected into animal models, IL-1 can also induce shock. However, it is unknown what discernible further roles it plays at this stage. TNFα, IL-6, IL-8, PAF, prostaglandins and leukotrienes all affect blood vessels causing abnormal endothelial function, such as constriction or relaxation. Complement is activated, and component C5a causes the PMNs to stick to blood vessel walls where they may release lysosomal enzymes and produce extensive damage. Activation of the coagulation cascade produces small clots and a condition known a disseminated intravascular coagulation (DIC) where blood vessels are blocked and normal blood flow inhibited, causing a dramatic drop in blood pressure (120, 121). The drastic drop in blood pressure results in a lack of oxygen flow to the major organs of the body such as the brain, heart and lungs. The high consumption of fibrinogen and platelets leads to an overall deficiency of available clotting components and leads to haemorrhages throughout the body. Acute respiratory distress syndrome (ARDS) is probably caused as a result of the coagulation cascade and is characterised by accumulation of fluid in the lungs causing insufficient gaseous exchange which is often the cause of death in septic shock sufferers (9, 71, 72, 74). Death due to septic shock has been reported to occur as little as 50 min to as much as 17 days post-transfusion (6, 137, 195).

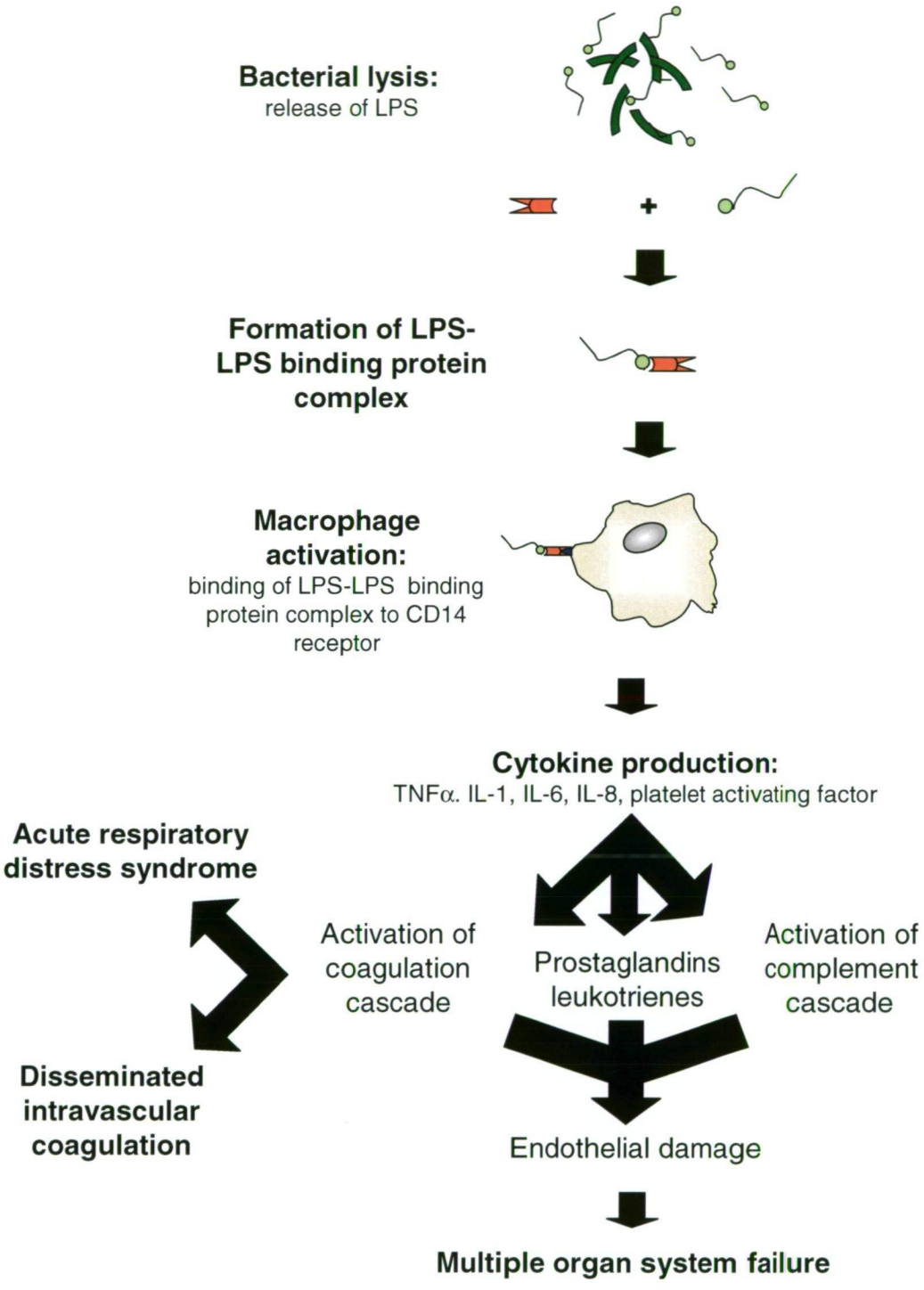


Figure 1.4: Schematic of LPS-induced sepsis

(Adapted from Salyers and Whitt, 1994)

Diagnosis and treatment of sepsis involves a peripheral blood smear which is examined for the absence of haemolysis. A Gram stain may provide similar information, although this technique is unreliable with less than 10^6 CFU/mL present in the sample (See also Section 1.7.3). An elevated WBC count would similarly suggest a tentative diagnosis of sepsis (86). Treatment must begin immediately, even prior to the identification of the organism responsible, with broad-spectrum antimicrobials of sufficient strength to counteract the most likely organisms, such as *Y. enterocolitica* and *Pseudomonas* spp. in RCCs and Gram-positive species in PCs (215). For RCC recipients, the most common treatment is an antipseudomonal penicillin in conjunction with co-trimoxazole to cover any possible *Y. enterocolitica* infection, while PC recipients commonly receive vancomycin and ceftazidime, particularly if the Gram-stain is negative (86, 214, 215, 227). It is, however, important to note that there have been reports of deaths following apparent recovery, and even when antibiotics have been applied as a prophylactic measure.

1.6.3 Incidence of transfusion-transmitted infections

The incidence of each microbial contaminant varies from country to country, depending upon the screening regime (including the donor questionnaire), the phlebotomy technique, and the antiseptic employed. However, in light of the data published by Chiu (49) and findings published since from the BaCon, SHOT and French haemovigilance studies, (63, 83, 116, 118, 175) indicating bacterial septicaemia is the most common transmitted infectious risk in transfusion medicine today, it is clear that the overall problem of TTBS is considerable (Table 1.4). These report between 10 to 72 PC and 0.2 to 5.8 RCC TTBS incidents, for every million transfusions conducted (35, 6, 81, 109, 132).

However the issue of underreporting (further explored in Sections 1.6 to 1.6.4) has led to suggestions that as many as 750 deaths per year occur as a result of TTBS in the USA alone (103). Unfortunately, there is a lack of equivalent Australian data for comparison. TTBS is also likely to be underreported for reasons as follow.

Table 1.4: Estimated current risk of transfusion transmitted infections per unit transfused

(from Menitove, 1996, Goodnough, 2003, Kiely, 2004, and Brecher, 2005)

Infection	USA
Viruses	
Hepatitis B	*USA*: 1 in 58 000 to 149 000 *Australia*: 1 in 1 339 000
Hepatitis C	*USA*: 1 in 872 000 to1 in 1.7×10^6 *Australia*: 1 in 3 636 000
HIV-1	*USA*: 1 in 1.4×10^6 to 1 in 2.4×10^6 *Australia*: 1 in 7 299 000
Bacteria	
PC	*USA*: 1 in 350 to 1 in 2000 *Australia*: Not available
RCC	*USA*: 1 in 1 000 000 *Australia*: Not available

1.6.4 Scope of transfusion-transmitted bacterial septicaemia

The symptoms of TTBS closely resemble other transfusion episodes, such non-haemolytic transfusion reactions, and hence TTBS may be misdiagnosed (132). Cross-matching of the isolate cultured from the recipient, blood product, and the donor provides a definite connection of the contaminated product to a case of TTBI. Unfortunately, this process can be significantly impeded. Although TTBS may be suspected, the organism responsible may not be cultured from either the patient or the product, or the product may be discarded before a culture can be made. Negative cultures from patients are most likely due to the prophylactic treatment of patients with antibiotics, as the bacteria may be non-viable (although their remnants continue to stimulate the patient's immune system). Moreover, as the organisms implicated in TTBS are frequently skin flora, they are often thought of as being insignificant when isolated in a clinical setting from patient blood cultures. Muder (139) reported an atypical case such as this, where the primary physician dismissed two repeated blood cultures of *S. epidermidis,* and remained unconvinced of the significance of this result until the same microorganism was cultured from a third blood sample.

Moreover, catheters and tubing are prone to bacterial contamination and the formation of biofilms (6). These items and procedures may therefore be incorrectly implicated as the source of the causative agent of a septic reaction, rather than the transfusion product. Finally, the most substantial reason for the postulated underreporting of TTBS is attributable to antibiotics. These are frequently prescribed to prevent opportunistic bacterial infections in transfusion recipients as most of these recipients are immunocompromised (53). This practice, although preventing spontaneous cases of infection, may also mask episodes of TTBS. However, the blanket application of prophylactic antibiotics to transfusion recipients may not be the simple solution to the TTBI problem, as is popularly thought. There are three reasons for this. Firstly, as 50% of organisms implicated in sepsis are Gram-negative, they can subsequently acquire resistance to some antibiotics, especially in a hospital setting. There has also been a documented increase in the antibiotic resistance of Gram-positive organisms. So while antibiotic treatment may be considered effective today, an increase in antibiotic resistance by many common bacterial pathogens has left patients susceptible to acquiring untreatable infections (25). Vancomycin-resistant *S. aureus* (VRSA) were isolated from a hospital

environment in 2002 (161). As this group of bacteria is one of the most common contaminants of PCs, its entrance into the blood banking system is highly likely. Secondly, TTBS mortality has been recorded despite the prophylactic application of antibiotics (as mentioned in Section 1.6.2). Finally, other considerations such as antibiotic toxicity and expense must also be taken into account.

Clearly, the problem of bacterial contamination is multifaceted and complex, and cannot be solved by prevention of contamination or suppressing bacterial growth, or monitoring for product spoilage. Moreover, it is difficult to accurately and quickly diagnose sepsis in a recipient (which has contributed to the underreporting of incidents), a situation which may rapidly lead to their death. The viral screening in place today reflects the serious nature and consideration of the viruses concerned. However bacterial contamination and transmission via blood products is considered with much less scrutiny although recent evidence suggests that the true scope and magnitude of this is much larger than the viral problem. In consideration of the limited bacterial screening of products and the potential risk to recipients, the need for additional screening methods has been raised.

1.7 Methods available for the detection of bacterial contaminants

The detection of bacterial contaminants in blood components is a complex problem, and a screening test to detect all relevant bacterial contaminants has strict requirements, as follows.

1.7.1 Requirements of an `ideal' test

There are four basic requirements for an ideal test:

1. **Specificity**: The false-positive and false-negative rates should be significantly lower than the estimated rate of sepsis.
2. **Sensitivity**: The test should be able to detect bacteria at lower concentrations than the numbers needed for a septic reaction ($<10^4$ CFU/mL) (133).
3. **Speed**: The test must be rapid enough for timely product clearance and distribution.
4. **Simplicity**: The results should be easy to interpret, with little or no instrumentation required. The test itself should be simple to perform, with as few steps as possible to minimise operator error.

In addition to these four key elements, the test should not be prohibitively expensive, and should be able to detect a wide range of bacteria in all blood product types (133, 200). The common screening methods available today will now be discussed and assessed as to how adequately they fulfil each of these requirements. Following this, more recent methodologies proposed for investigation in this thesis will be reviewed.

1.7.2 Visual inspection of products

Detection of bacterial contamination by simple visual inspection is particularly appealing as it requires no equipment. There is no expense and the technique is non-invasive (133). A number of features which are different for each of the main products of concern, RCCs and PCs, can be utilised to determine the presence of bacterial contamination.

1.7.2.1 Red cell concentrates

Kim (112) noted that when RCCs were deliberately contaminated with *Y. enterocolitica*, the culture-positive main bag section of the product was darker than attached side tube segments which were culture-negative. The same phenomenon has been observed with a range of other species (110). Kim was able to demonstrate that haemolysis was responsible for the darkening colour, and this became apparent upon visual inspection with contaminating numbers of bacteria greater than of 10^8 CFU/mL (110). Pickard (156) reported that trained personnel could detect spoilage from bacterial contamination with as few as 10^4 CFU/mL present with 97% accuracy, when the blood pack side tubing was considered in comparison to the main bag. However, the accuracy dropped to 47% when the blood pack main bag was considered on its own. Pickard's observations were unreliable, however, as some positive identifications were only able to be made when bacterial numbers peaked at 10^9 CFU/mL, whilst some uncontaminated packs were identified as contaminated (156). The unreliability of this technique has been well documented. In the two years prior to 1992 in the USA, there were seven fatalities involving *Y. enterocolitica*. Of these fatalities, most were following the transfusion of RCCs that showed no gross evidence of contamination (7, 105, 192). Clearly this bacterial detection technique when used alone is unreliable and insensitive, but there are

additional limitations beyond these basic problems. The technique of comparing the blood bag with side tubing prior to transfusion can only be applied to RCCs, and therefore is unsuitable as the sole method of detection for bacterial contamination. It may, however, be used in tandem with other detection methods such as automated culture.

1.7.2.2 Platelet concentrates

A phenomenon known as 'swirling' or 'streaming' is associated with normal platelet discoid morphology and is used to evaluate each PC qualitatively for viability (18). This is done by tapping or inverting the PC product during exposure to a light source, whereby normal platelets will reflect the light, whereas non-discoid platelets will not (133). Wagner and Robinette (203) observed that the swirling effect ceased to be observed at bacterial concentrations of 10^7 to 10^8 CFU/mL. Clotting, small white clumps and a light green colour were also seen in some instances. Further investigation revealed the cessation of the swirling effect was attributable to a decrease in pH produced as a result of the metabolism of the bacteria. However, bacterial contamination is not the only cause of the loss of swirling. Bertolini (18) and Wagner and Robinette (203) demonstrated independently that a number of other factors also contribute to the lack of swirling, including prolonged storage at 22°C and lower storage temperatures. The latter group clearly indicated that up to 18% of PC showed no swirling effect by day 5 of storage at normal PC storage conditions. This would result in an unacceptably high level of false-positives, (even though these non-swirling PC may not be an effective therapeutic product if they were transfused to a recipient).

In conclusion, the evaluation of PCs for bacterial contamination by swirling, clotting, or colour changes, must be considered in the same light as evaluation of RCCs by pack darkening. Both are relatively insensitive techniques on their own. Due to this, PC examination is used with other detection techniques, such as automated culture.

1.7.3 Staining: Gram and acridine orange

Staining techniques explored in the detection of bacterial contaminants of blood products include Gram stain and acridine orange. Reik (165) found that at concentrations of 10^5 CFU/mL in buffy coat smears, approximately 7 microorganisms were visible on Gram staining in a single high power field of view on close inspection. At 10^6 CFU/mL, 50 to 100 microorganisms were apparent at the same magnification. This variance may be attributable to the differential clumping ability of each individual organism, or properties of products themselves. This is supported by Yomotovian (225) who cites a case of a contaminated PC unit with more than 10^7 CFU/mL of *P. aeruginosa* that was deemed negative, a result that was attributed to the clumping nature of the organism. They further determined that true positive results with PCs were only reliable with contamination levels of 10^6 CFU/mL or more, and that sensitivity prior to day 4 of storage was only approximately 80%. Hence, unreliability and relative insensitivity appear to be the greatest limiting factors in the implementation of this technique for routine screening for contaminants. However, as it is relatively inexpensive and quick, it is used overseas in some blood centres to reduce the likelihood of transfusing highly contaminated products (4).

Acridine orange is used to improve the sensitivity of detection. Kim (113) reported that they had consistently been able to detect *Y. enterocolitica* in RCCs at concentrations between 10^4 to 10^5 CFU/mL. There is some debate as to whether this sensitivity applies to all organisms and all products. Chongokolwatana (51) reported a 100% detection success rate only with more than 10^6 CFU/mL, and an 83% detection success rate with bacterial concentrations of 10^5 to 10^6 CFU/mL. Therefore, acridine orange appears to suffer from similar drawbacks to the Gram stain in terms of sensitivity. Additionally, it requires a fluorescence microscope for visualisation of the bacteria.

In conclusion, this technique has its place as an interim pre-transfusion detector of contaminants. However, as there appears to be no conclusive evidence supporting the greater sensitivity of acridine orange over the Gram stain, and there is the requirement of additional detection equipment for the former, it may be more appropriate and cheaper to utilise the Gram-stain for routine screening.

1.7.4 *Limulus* amoebocyte lysate assay

The *Limulus* amoebocyte lysate (LAL) assay has been extensively described in the literature and detects bacterial endotoxin (7, 112). Arduino (7) detected endotoxin in RCCs produced by bacteria of concentrations between 10^1 to 10^5 CFU/mL. However one unit of a concentration of 10^3 CFU/mL was not detected for reasons unknown. This test is limited to detection of endotoxin-producing Gram-negative species and the specificity and sensitivity of the test appear unreliable. Hence, it is unsuitable as a stand-alone method for pre-transfusion product testing (7, 193). As for the previously described techniques (Sections 1.7.1 to 1.7.3), it may be used in conjunction with other interim screening measures as an indicator of bacterial contamination, rather than as an absolute screening method.

1.7.5 Short-term culture

Short-term culture is conducted by sampling PCs at day 3 of storage, and detection of bacterial growth is facilitated by an automated culture machine (120). Blachman (27) found that of 16 290 PCs tested the day after manufacture, only 1 positive PC was detected, but by the third day another 4 PCs were positive. A more recent study sought to expand these original experiments and determine whether sampling could be conducted at 24 or 48 hours post-inoculation, and how long a positive detection would take after these time-point samplings. Wagner and Robinette (202) found with *S. epidermidis* (0.1 CFU/mL inoculum) 24 hour post-inoculation samples, that these took an average of 24 to 37 hours to become positive by BacTAlert. If sampling occurred 48 hours post-inoculation, the system required 11 to 17 hours for detection. Therefore, it would still take up to 3 days to make a positive detection, which would allow for substantially less time for PCs to be transfused, if they had to be cleared by short-term culture. In addition to the slow speed at which automated culture operates, the false-positive rate can vary considerably (Section.1.4.4). Although more rapid, short-term culture suffers from many of the drawbacks of conventional culture and is generally not considered practical for application as a screening measure for all products. However, despite these limitations, the USA, Belgium, the Netherlands, and most blood centres in Sweden, Norway and Denmark have introduced mandatory short-term culture of PCs to extend the shelf life of culture-negative PCs to 7 days (3).

Clearly, the development of the next generation of bacterial screening assays is warranted, utilising more sophisticated techniques than those in place today. The following section will attempt to define the targets most likely to be of use in a detection method for bacterial contaminants, and any potential limitations they may present.

1.8 Detection methods to be investigated in this study

Detection of bacterial contaminants at the nucleic acid level has been pursued with particular vigour since the successful development and introduction of HIV and HCV NAT tests. The detection of the bacterial nucleic acid has been directed along two major avenues. That being species-specific targeting using discrete genetic elements or unique regions of the bacterial ribosomal RNA (rRNA) gene, or targeting sequence common to multiple bacterial species, such as conserved regions of the rRNA gene.

1.8.1 Bacterial 16S ribosomal genes

Until relatively recently, the identification of microorganisms was based solely on culture, biochemical and serological tests. However, increasingly it was being found that many species could not be successfully cultured or classified based on these properties alone. 16S ribosomal genes are found in all bacteria, and these contain conserved and variable regions (Fig 1.5) (221). In the variable regions, slow evolution over time leads to mutations which give rise to new signature sequences, which can be used to identify novel bacterial species or subsets, without the need for culture (166-168, 220). The conserved regions are consistent in all bacterial species, due to structural constraints needed for the rRNA molecule to function. By using both conserved and variable regions, it has been possible to design broad-range and specific primers or probes to detect these sequences (219).

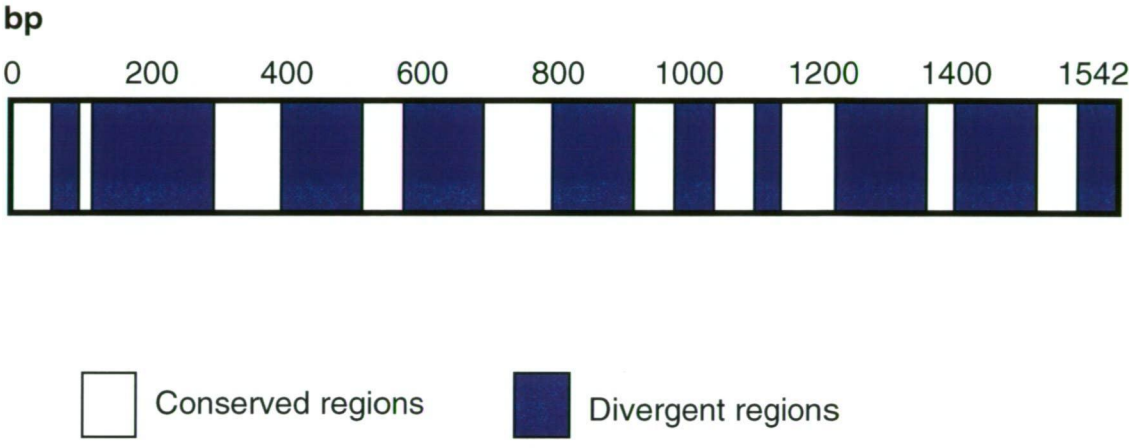


Figure 1.5: Schematic diagram of the bacterial 16S rRNA gene

1.8.2 Polymerase chain reaction (PCR)

Feng (70) was first to demonstrate the application of the polymerase chain reaction (PCR) to *Y. enterocolitica* in WB. Amplification of the correct *Y. enterocolitica* sequence was confirmed by Southern hybridisation, and PCR sensitivity was estimated at 5×10^3 CFU/mL. Although the sensitivity and simplicity of the PCR tests satisfy the requirements of the 'ideal test', (see Section 1.7.1) the approach used by this group enabled amplification of just one species out of a large spectrum of bacteria that contaminate blood products. Of more practical use, generic 16S PCR gives the ability to detect all bacteria using primers targeted at conserved sequences. It has been successfully used to detect bacteria in clinical (60, 124, 188, 210, 219) and environmental samples (87), and may therefore provide a means for detecting the diverse array of bacteria found in blood products.

There are several issues with the PCR detection of bacteria in blood products. The detection takes approximately 6 hours, which means it cannot be used immediately prior to transfusion although it may be applied at the start of each day during periods of low demand. Confirmation of PCR product sequence identity by Southern dot blotting or sequencing, can take at least another 24 to 48 hours. Moreover, contamination of the PCR reaction ingredients by ubiquitous environmental bacterial DNA, leave the test vulnerable to false-positive reactions. Blood products have also been found to contain proteins that interfere with the PCR sensitivity and hence this problem must also be overcome (1, 62, 152, 218). Furthermore, if universal 16S sequences are targeted, it will be impossible to identify if one or more bacterial species is present. The cost of PCR detection is also relatively expensive compared to other detection techniques, and operator proficiency is required for reliable performance of the test.

1.8.3 Real-time polymerase chain reaction (real-time PCR)

Real-time PCR is increasingly being used in preference to standard PCR as a diagnostic tool. While similar in theory to standard PCR, real-time PCR incorporates the use of generic or specific fluorescent probes within the mastermix, which eliminate the need for electrophoresis and staining. The primers target shorter sequences (usually about 150 bp) than those generally used for standard PCR, as this increases the speed at which the assay can be performed. Amplification and positive

detection can be completed in as little as 30 min. As the reaction progresses, fluorescent markers are incorporated into the double-stranded amplicon, and the fluorescent signal (detected by laser) increases as the amount of product increases. Two kinds of reporters are used. SYBR green is most common, and is a generic method of quantifying the PCR product. It non-specifically binds to the major groove of DNA (similar to ethidium bromide), hence the more product, the greater the fluorescence, although this method cannot confirm the identity of the PCR product. A surrogate method termed a 'melt profile' has been developed to solve this problem. Each amplicon has a signature melt profile, based on its sequence (similar to the size of an amplicon as it is electrophoresed through an agarose gel). At the end of each real-time PCR run, the samples are gradually heated over a range of temperatures until the product melts and the fluorescent signal is extinguished. This temperature becomes the signature for the amplicon, and subsequent real-time PCRs can be judged positive (or negative) on the basis of this melt profile. Fluorescently-labelled oligonucleotide probes can also be used, and these specifically bind to discrete genetic sequences within the amplicon. These can simultaneously confirm the identity of the amplicon as the reaction progresses. Applications using real-time PCR as a diagnostic assay for infectious diseases have been recently published, and have shown encouraging results (89, 153, 187). However, the small size of the targets and the ubiquitous nature of bacterial DNA in the environment and PCR reagents may result in this technique being vulnerable to false-positive results if applied using generic 16S sequences. The cost of real-time PCR (particularly with the fluorogenic probes) is significantly greater than the cost of standard PCR. However, as the use of real-time PCR increases, costs are likely to be greatly reduced.

1.8.4 Polymerase chain reaction-enzyme linked immunoassay (PCR-ELISA)

A molecular-based ELISA was developed as an adjunct to the PCR process, by which amplicons could be identified using highly specific, biotinylated capture probes. In this procedure, DIG-labelled PCR product samples are hybridised to biotinylated probes, which in turn bind to streptavidin coated 96-well microtitre trays. A horse radish peroxidase (HRP) conjugated anti-DIG antibody is added to each well, and binds to the DIG-labelled samples. A substrate is added and is

cleaved in the presence of the HRP, leading to colour production, which can be measured by spectrophotometer. An advantage of this method is that it may be fully automated, utilising existing infrastructure available at the ARCBS. Furthermore, it is rapid enough that testing may be carried out centrally at the ARCBS upon receipt of a bulk product order, and the test-negative products may be released for use the same afternoon. Also, the overall complexity of the assay is no greater than a standard ELISA (such as the ChLIA (Section 1.4.1) which is currently used at the ARCBS).

This method has been successfully used in a range of applications, including the detection of viral pathogens (such as human papillomaviruses and parvovirus B19) (158, 228), the identification of overexpressed T cell receptor genes in blood and tissue biopsies (198), and in identifying a range of bacteria such as *Mycobacterium tuberculosis* (30), *Listeria monocytogenes* (184) and *E. coli* (61) in food and clinical samples. Using a pre-enrichment step, these tests have been found to have a sensitive and specific detection limit of 1 to 5 CFU per 25 g of food or 10^2 CFU/mL of milk. However, all instances using PCR-ELISA to detect bacteria have utilised amplicons generated from specific gene targets. A universal 16S PCR, followed by probing of unique sequences within this region, would enable the detection of a range of bacteria using a single protocol. It is this approach that will be explored in this thesis

1.8.5 Southern dot blot

Southern dot blotting is a technique by which unknown sequences can identified through hybridisation with a probe of a known sequence. It can be performed in two orientations: with the DIG-labelled probe of known sequence in solution, and the unidentified sample bound to a membrane (standard orientation), or whereby the DIG-labelled sample is in solution, with the probes of known sequences bound to the membrane. Each has a unique advantage over the other. The standard orientation allows for many samples to be examined with a single probe, whilst reverse-probing allows a single sample to be tested against many probes simultaneously (similar to the PCR-ELISA approach). This procedure may be fully automated and conducted at a central testing point, which added to its appeal as a potential detection method. Our aim in this study was to use small oligoprobes to

target and identify small sequence differences between our bacterial 16S PCR products, in order to identify the bacterium responsible.

1.9 Summary and objectives of the project

The problem of bacterial contamination of blood products is a long-standing one. Yet it has only been in recent times (and with the success of the viral screening programs) that the problem of transfusion-transmitted bacterial sepsis (TTBS) has come into greater focus. In Australia, the incidence of TTBS is poorly defined. Elsewhere it is underreported, but is known to cause significant morbidity and mortality in the hospital setting. The methods in place today for detection of bacteria in blood products rely on culture-based methods, which are slow, labour intensive, susceptible to false results, and furthermore are only performed after the expiry of the blood product. This method is impractical for routine screening of all blood products and consequently, just 1% of each kind of blood product produced is screened for bacteria in Australia.

This study will define the scope and nature of the bacterial contamination problem in Australia, and seek a possible solution for practical screening of blood products. The major aims of this project are as follows.

1. To further identify those microorganisms most likely to be a problem in product spoilage and TTBS by comparing the growth characteristics of these bacteria in buffy coat blood products.
2. To establish and develop a detection method that can be applied to blood and blood products and evaluate its use in the detection of bacterial contamination in such products.
3. To evaluate the efficacy of this method on 'real' samples with bacteria grown in PCs and RCCs from the growth kinetic experiments, as compared with spiked product samples.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Introduction

This chapter describes the materials and experimental techniques used to study the growth kinetics of bacterial contaminants in blood products (Chapter 3), the extraction of bacterial DNA from blood products (Chapter 4), and the detection of bacterial contaminants by molecular methods (Chapters 4 and 5). Methods that are not specifically described were performed as detailed in Current Protocols of Molecular Biology (11), or as stated by Sambrook (179).

2.2 Materials

Materials and equipment used during the course of this work were obtained from the companies listed in Table 2.1.

2.2.1 Chemicals

Analytical grade chemicals were used without further purification. Solutions were routinely prepared with deionised distilled water (ddH_2O) and sterilised by autoclaving at 121°C for 20 min or passage through a 0.22 µm pore size filter (Millipore).

2.2.2 Bacteriological media and culture conditions

The manufacturers of the bacteriological media used in this study are presented in Table 2.1. Bacterial culture media were prepared as per the manufacturers' instructions, with the addition of 1.5% (wt/vol) agar (Oxoid) to broths when solid media were required. Sterile ddH_2O was used to make all media, which were prepared as follows: (i) Trypticase broth: 3.0% (wt/vol) Tryptone soya (Oxoid) (160); (ii) Brain heart infusion (BHI): 3.7% (wt/vol) Brain-heart infusion (Oxoid); Horse blood agar (HBA) plates (containing 5% (vol/vol) washed horse erythrocytes) were purchased from Taslab Services (Launceston) and the University of Melbourne Media Department (Parkville).

All bacteria were routinely cultured at 37°C in BHI or trypticase broth with shaking (*Y. enterocolitica* and *P. fluorescens* were grown at 30°C).

Table 2.1: Suppliers of materials and equipment used in this study

Company	City/State	Country
Amersham	Little Chalfont, Buckinghamshire	United Kingdom
Applied Biosystems	Foster City, CA	USA
ARCBS	Hobart, TAS and Melbourne, VIC	Australia
Baxter Fenwal	Deerfield, IL	USA
Baxter Fenwal	La Châtre	France
BDH	Dorset	United Kingdom
Becton Dickinson	Meylan Cedex	France
Bio Mérieux	St Louis, MO	USA
BioRad	Hercules, CA	USA
Briemar Nominees	Kooweerup, VIC	Australia
Corbett Research	Sydney, NSW	Australia
CSL Ltd	Parkville, VIC	Australia
Difco Laboratories	Detroit, MI	USA
Eastman Kodak Co.	Rochester, NY	USA
Geneworks Pty Ltd	Adelaide, SA	Australia
ICN Biomedicals	Seven Hills, NSW	Australia
Invitrogen	Carlsbad, CA	USA
Microsoft	Redmond, WA	USA
Millipore	Bedford, MA	USA
New England Biolabs	Beverly, MA	USA
Nunc	Roskilde	Denmark
Oxoid	Basingstoke, Hampshire	United Kingdom
Pharmacia	Madison, WI	USA
Proligo	Boulder, CO	USA
Promega	Madison, WI	USA
Qbiogene BIO101	Carlsbad, CA	USA
Qiagen	Hilden	Germany
Ratek Instruments	Boronia, VIC	Australia
Roche Applied Science	Basel	Switzerland
Sarstedt	Adelaide, SA	Australia
Sigma Chemical Co.	St. Louis, MO	USA
Spectronics Corporation	Westbury, NY	USA
Terumo	Tokyo	Japan
Themo Savant	Holbrook, NY	USA
World Precision Instruments	Sarasota, FL	USA

2.3 Bacterial strains

The bacterial strains used in this study are listed in Table 2.2.

2.4 Blood products

PCs and RCCs were produced by the ARCBS using the buffy coat method according to the Council of Europe standard procedures (2003), as shown in Fig 1.1. Approximately 70% of PCs and RCCs obtained for this study were prepared from blood group B individuals, and about 25% were from group A individuals. In brief, 450 mL of whole blood was collected into 'top-and-bottom' Optipacs (Baxter Fenwal, France) containing citrate-phosphate-dextrose anticoagulant. Following centrifugation, the RBCs (PL 146 bags - Baxter Fenwal, USA) and plasma were expressed into separate satellite bags using an automated blood component separator (Optipress, Baxter, USA) leaving the platelet-rich buffy coat in the primary pack.

2.4.1 Platelet concentrates (PCs)

PCs were prepared by pooling five units of ABO/Rh matched buffy coats, and the platelet rich fraction was transferred to a PL 2410 bag (Baxter Fenwal, USA) and resuspended in 300 mL T-sol® platelet additive solution (Baxter Fenwal, USA). The final PC products contained approximately 8×10^7 WBCs/unit. PCs were stored under standard conditions (22 ± 2°C with agitation) for 7 d.

2.4.2 Red cell concentrates (RCCs)

RCCs were resuspended in 100 mL Adsol solution, and contained around 10^8 WBCs/unit. RCCs were stored under standard conditions (4 ± 2°C, static) for 42 d.

Table 2.2: Bacterial species and strains used in this study

Species	Strain	Reference/Source
Bacillus cereus	ATCC 11778	ATCC[a]
B. cereus	B99343476	This study[b]
B. subtilis	ATCC 6633	ATCC[a]
Corynebacterium diphtheriae	ATCC 13812	ATCC[a]
Enterobacter aerogenes	ATCC 13048	ATCC[a]
Enterobac. aerogenes	98401532	This study[b]
Enterobac. cloacae	-	(199)[c]
Enterobac. cloacae	B98347611	This study[b]
Enterococcus faecalis	ATCC 29212	ATCC[a]
Enterococ. faecalis	97683909	This study[b]
Enterococ. faecium	ATCC 35667	ATCC[a]
Escherichia coli	ATCC 25922	ATCC[a]
E. coli	ATCC 35218 (β-lac+)	ATCC [a]
E. coli	B97680290	This study[b]
E. coli	Plasma resistant	This study[b]
E. coli	W	(199)[c]
Klebsiella oxytoca	-	This study[a]
K. pneumoniae	ATCC 700603	ATCC [a]
K. pneumoniae	B98347672	This study[b]
Pseudomonas aeruginosa	ATCC 27853	ATCC [a]
P. aeruginosa	98395615	This study[b]
P. fluorescens	B49406340	This study[b]
Salmonella Typhimurium	ATCC 14028	ATCC[a]
Salm. Typhimurium	5850	This study[b]
Serratia marcescens	-	(199)[c]
Serr. marcescens	W	(199)[c]
Coagulase negative staphylococcus	-	This study[b]
Staphylococcus epidermidis	ATCC 12228	ATCC[a]
S. epidermidis	98350139	This study[b]
S. aureus	ATCC 25923	ATCC[a]
S. aureus	ATCC 29213	ATCC[a]
S. aureus	ATCC 43300	ATCC[a]
S. aureus	2001:04:03 (MRSA)	This study[a]
S. aureus	B98347745	This study[b]

(Cont.) Table 2.2: Bacterial species and strains used in this study

Species	Strain	Reference/Source
S. aureus	Workshop isolate 57	This study[a]
S. saprophyticus	ATCC 35552	ATCC[a]
S. warneri	B98337830	This study[b]
group A streptococcus	ATCC 19615	ATCC[a]
group A streptococcus	M629137	This study[b]
group B streptococcus	ATCC 13813	ATCC[a]
group B streptococcus	MCRI 27	This study[a]
Strep. mitis	B98347705	This study[b]
Strep. pneumoniae	ATCC 6305	ATCC[a]
Strep. pneumoniae	ATCC 49619	(104)[a]
Strep. pneumoniae	98339025	This study[b]
Vibrio cholerae	6239	This study[b]
Yersinia enterocolitica	ATCC 23715	(188)[a]
Y. enterocolitica O3, biovar 4	67R (pYV+)	(171)[b]
Y. enterocolitica O3, biovar 4	67W (pYV-)	(171)[b]
Y. enterocolitica O3, biovar 4	-	(199)[c]
Y. enterocolitica O8, biovar 1B	8081	(157)[b].
Y. enterocolitica O8, biovar 1B	CHOC (pYV+)	(21)[b]
Y. enterocolitica biovar 1A	T83	(82)[b]
Y. enterocolitica O1,3, biovar 3	AC27 (pYV+)	This study[b]
Y. enterocolitica O5,27, biovar 2	IP885 (pYV+)	(209)[b]
Y. enterocolitica O5,27	CIDC2064	This study[b]
Y. enterocolitica O6,31, biovar 1A	CIDC6407	This study[b]
Y. enterocolitica O7,8	IP106	This study[b]
Y. enterocolitica O9	W22703 (pYV+)	(13)[b]
Y. frederiksensis	Q62	This study[b]
Y. kristensis O11	WS60/88	(170)[b]
Y. pseudotuberculosis O2b	314698	This study[b]

[a] Bacteriology Laboratory, Royal Children's Hospital, Parkville, Australia.
[b] Roy Robins-Browne, Department of Microbiology and Immunology, University of Melbourne, Parkville, Australia.
[c] Rosemary Sparrow, Australian Red Cross Blood Service – Victorian Division, Melbourne, Australia.

2.5 Spiking and sampling of blood products

All bacteria and products used in this thesis were prepared in the following manner. At least two separate experiments were conducted for each bacterial species and product type.

2.5.1 Preparation of cultures

In preparation for product spiking, bacteria were harvested by centrifugation (3000 ×*g* at room temperature, RT), washed twice with sterile PBS, and resuspended in 5 mL PBS. The number of bacteria per mL was estimated by measuring samples at OD_{570} and comparisons with standard curves, and also confirmed by plate counts. The bacterial suspension was diluted to provide final concentrations of approximately 10, 10^2 or 10^3 CFU/mL in products when spiked with 1 mL of suspension. Spiking doses were selected to reflect the levels of contamination that may be anticipated in blood products at donation (45, 206). Products were initially spiked with 10^2 CFU/mL, and the experiment repeated with a higher (10^3 CFU/mL) or lower (10 CFU/mL) number of bacteria, depending on whether proliferation was observed with the first spiking concentration.

2.5.2 Preparation of blood products

For the spiking experiments, PCs were divided into 5 ×50 mL aliquots in PL 1240 bags (Baxter Fenwal); 20 mL was kept in the original bag as a control. Spiked PCs (each containing approximately 1.6×10^7 WBCs/pack) and control samples were held under standard PC storage conditions for 7 d. Five replicate RCCs were obtained, spiked, and held under standard storage conditions for 36 d (56). This shortened storage period (rather than the 42 d that is standard) was used as previous studies have found that bacteria that proliferate in stored RCCs do so before this time point (37, 100). A 20 mL portion from each RCC pack was kept under the same conditions as a negative control.

2.5.3 Spiking procedure

As bacteria can be easily introduced to the blood products through poor handling procedures, gloves worn for experimentation were prepared with 70% ethanol, and scissors used for cutting were treated with a sterile swab of 70%

isopropanol (Briemar). The side tubing of the blood bag was also prepared aseptically with a sterile swab of 70% isopropanol. A 1 mL volume of the prepared bacterial suspension was infused into each of the replicates, the tubing re-sealed by an Ultrasonic Tube Sealer (Terumo), and a sterile coupling device (Baxter Fenwal) was attached to the side tubing for ease of repeat sampling. The pack was inverted several times to ensure complete bacterial dispersion. Before sampling, the sterile coupling device was aseptically prepared by 70% isopropanol swab, and then a 1 mL sample was withdrawn using a 3 cc syringe (Terumo). Plate counts were performed on each replicate using 100 µL duplicate samples to ensure accuracy of spiking. The negative control was sampled to confirm sterility at 0 d.

2.5.4 Sampling of spiked and control products

Spiked and control PCs were sampled (1 mL) aseptically at 24 hour intervals for 7 d. Spiked and control RCCs were sampled (1 mL) on d 0, 1 and 3, and then at 3-d intervals to 36 d. While bacterial numbers were less than 10^4 CFU/mL, aliquots of 100 µL were spread in duplicate on BHI agar and incubated as described above. As bacterial numbers increased, 1 in 10 dilutions of 100 µL samples were made in PBS and spotted onto BHIA in 10 µL duplicate aliquots. Any colour and physical changes (such as clumping) of blood products indicative of spoilage were also recorded. For RCCs, a positive spoilage result was recorded when pack darkening (indicative of RBC haemolysis) occurred. For PCs, a loss of swirling, an increase in product coudiness, or platelet clumping identified the spoiled components.

2.6 DNA purification techniques

2.6.1 Qiagen QIAMP DNA Mini Kit

Bacterial extractions were conducted as per the manufacturer's instructions for Gram-positive bacteria (Protocol D). Purified DNA was eluted in 100 µL AE Buffer (supplied by the manufacturer) at 4°C.

2.6.2 BIO101 FastDNA SPIN Soil Kit

The manufacturer's extraction method was followed, and purified template was eluted into 100 µL ddH_20 for storage at 4°C.

2.6.3 Novel rapid extraction method: bead-beating with spin column purification

Bacteria were grown in 10 mL BHI broth with shaking at 37°C (except *Y. enterocolitica* and *P. fluorescens*, which were grown at 30°C) for 16 h and then pelleted by centrifugation at 3 500 ×*g* for 5 min. The supernatant was removed and the pellet resuspended in 200 µL lysis buffer (0.2 M $NaH_2PO_4 \cdot H_2O$, 0.2 M $Na_2HPO_4 \cdot 7H_2O$ pH 7.0) (222), prior to being added to a 500 µL screw-cap tube containing 120 µL 250 micron glass beads (Sigma) and 25 µL MT buffer (Qbiogene). Samples were bead beaten for 180 s at setting 5.5 in a FastPrep FP101 (Qbiogene ThermoSavant) to maximise cell lysis and DNA recovery. Machine cooling time (5 min) was allowed after 90 s. The tubes were briefly chilled on ice, and then centrifuged for 5 min at full speed to pellet large cellular debris. The recovered supernatant was placed in a fresh microfuge tube, to which 40 µL of Proteinase K (Qiagen, supplied with the MiniKit) was added, the samples vortexed, and then incubated for a minimum of 1 h, with occasional mixing. Samples were centrifuged 5 min at 12 000 ×*g*, and the supernatant placed in a fresh microfuge tube, to which 75 µL of potassium acetate (7.5 M) were added per 200 µL of recovered supernatant. Samples were vortexed vigorously for 15 s and centrifuged 5 min at 12 000 ×*g* to precipitate the proteinaceous content, before the supernatant was removed to a fresh microfuge tube. Two hundred microlitres of RT 100% ethanol (BDH) were added to the supernatant and vortexed vigorously for 15 s. The purification of extracted DNA was carried out using columns and reagents from the Qiagen DNA MiniKit, according to the manufacturer's instructions. All centrifugation steps were carried out at RT at 12 000 ×*g*, unless otherwise indicated. In brief, an appropriate amount of AE Buffer (supplied with the kit) was pre-warmed to 70°C in a heat block (Ratek). Each sample (including any white precipitate) was added to a spin column, and centrifuged for 1 min. The spin filter was placed in a fresh spin column collection tube, and AW1 buffer (500 µL, Qiagen) was added.

Samples were centrifuged for 1 min, and the spin filter was placed into a fresh spin column collection tube. AW2 buffer (500 µL, Qiagen) was added to the column and the samples were centrifuged for 3 min. The spin column was placed in a fresh collection tube (not supplied with the kit), and re-centrifuged for 1 min to remove all traces of the AW2 buffer and to dry the filter. The spin columns were then placed in a fresh collection tube (not supplied with kit), and 100 µL of pre-warmed AE buffer was added directly to the filter and the samples incubated for at least 1 min at RT. Samples were then centrifuged for 1 min, and eluted DNA placed in a labelled screw-capped tube (Sarstedt) at 4°C for storage.

For the extraction of bacterial DNA from contaminated blood products, the following steps were added prior to the bead beating step in the above method: A 180 µL volume of pre-lysis buffer (20 mM Tris-Cl pH 8.0, 2 mM EDTA, 1.2% Triton-X 100) (Qiagen Gram-positive lysis buffer, as per the manufacturer's recipe) was added to each sample which was then vortexed vigorously for 10 s. Following centrifugation (3 min) the supernatant removed and the remaining pellet subjected to the above extraction procedure.

2.6.4 Estimation of DNA concentration

The absorbance of DNA at 260 nm was measured using an LKB-ultraspec-plus-spectrophotometer (Pharmacia). An absorbance of 1.0 was equivalent to a concentration of 33 µg/mL of single-stranded DNA and 50 µg/mL of double-stranded DNA.

2.7 Manipulation of DNA

2.7.1 Restriction endonuclease digestion

The restriction enzyme *Rsa*I was obtained from Roche Applied Science and used to cleave DNA (PCR product) in the following typical reaction as recommended by the manufacturer: 0.5 to 2 µg DNA, 5 to 10 units of enzyme, one tenth volume of restriction buffer, and the appropriate amount of sterile ddH_20 (20 to 30 µL). The reaction was incubated at 37°C for 1 h, followed by 15 min at 70°C to

inactivate the enzyme. Complete cleavage of the target was checked by gel electrophoresis.

2.7.2 DNA gel electrophoresis

DNA was analysed by electrophoresis on 1.0 to 2.0% (wt/vol) agarose (Roche Applied Science) gels, using a Tris-acetate-EDTA (TAE) buffer system (pH 8.0), and containing 0.5 μg/mL ethidium bromide to permit visualisation of the DNA on an ultraviolet (UV) light transilluminator. Prior to loading, DNA was mixed with sample loading buffer to give a final concentration of 10% (wt/vol) sucrose, 10 mM EDTA, and 0.01% bromophenol blue. Gels were run at 7.5 V/cm for 1 to 2 h, and then photographed using a Kodak Land Camera and Polaroid 667 film.

2.8 Polymerase chain reaction

2.8.1 Oligonucleotides

Oligonucleotides used in this study were manufactured by GeneWorks Pty Ltd, Proligo, Sigma or Invitrogen and are listed in Table 2.3. Oligonucleotide sequences were obtained from published sources where indicated, or were designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), and were analysed using the BLASTN Algorithm at The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence comparisons of the 16S rDNA gene between species were conducted when necessary using the ClustalW (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) program.

2.8.2 Standard PCR

The PCR method for the amplification of DNA was based on the method of Saiki (177). Template DNA was prepared as per Section 2.6.3. One to ten microlitres of template was used in each PCR. The 50 μL PCR typically contained 1 μM (each) oligonucleotide primer, 1 ×PCR buffer (10 mM Tris-HCl, 50 mM KCl) (Applied Biosystems), 250 μM (each) dNTP, 1.2 to 2.4 mM $MgCl_2$ (Applied Biosystems), and 1 U AmpliTaq GOLD DNA polymerase (Applied Biosystems).

Table 2.3: Oligonucleotides used during this study for PCR

Name	Target	Sequence (5'-3')	Reference
1537r	Human β-globin gene	ACCACTTTCTGATAGGCAGC	This Study
GH20	Human β-globin gene	GAAGAGCCAAGGACAGGTAC	(175)
KM29	Human β-globin gene	GGTTGGCCAATCTATCCCAGG	(175)
PC03	Human β-globin gene	ACACAACTGTGTTCACTAGC	(175)
PC04	Human β-globin gene	CAACTTCATCCACGTTCACC	(175)
RS40	Human β-globin gene	ATTTTCCCACCCTTAGGCTG	(175)
RS42	Human β-globin gene	GCTCACTCAGTGTGGCAAAG	(175)
RS80	Human β-globin gene	TGGTAGCTGGATTGTAGCTG	(175)
11A	644bp of the *Y. enterocolitica* pYV *virF* gene	AAGGTTGTTGAGCATTCACAAGATGG	(192)
12A	644 bp of the *Y. enterocolitica* pYV *virF* gene	TTTGAGTGAAATAAGACTGACTCGAGAACC	(192)
mec1	550 bp of the *mecA* gene	AAAATCGATAAAGGTTGGC	(15)
mec2	550 bp of the *mecA* gene	AGTTCTGGCACTACCGGATTTGC	(15)
A1	330 bp of the *ail* gene	TTAATGTGTACGCTGGGAGTG	(206)
A2	330 bp of the *ail* gene	GGAGTATTCATATGAAGCGTC	(206)
TStaG422	317 bp of the *tuf* gene	GGCCGTGTTGAACGTGGTCAAATCA	(125)
TStag765	317 bp of the *tuf* gene	TIACCATTTCAGTACCTTCTGGTAA	(125)
ymoACR	292 bp of the *ymoA* gene	GACTTTTCTCAGGGGAATAC	(83)
ymoaDF	292 bp of the *ymoA* gene	GCTCAACGTTGTGTGTCT	(83)
DG74	Universal 16S rDNA target: 313 bp with both 143 and 68d	AGGAGGTGATCCAACCGCA	(86)
143	Gram-positive 16S rDNA target	GAYGACGTCAARTCMTCATGC	(112)
68d	Gram-negative 16S rDNA target	AYGACGTCAAGTCMTCATGG	(112)
65ab	Universal 16S: 333bp with DG74	AACTGGAGGAAGGTGGGGAY	(112)
27f	1420 bp of the16S rDNA gene	AGAGTTTGATAATGGCTC	(115)
1492r	1420 bp of the16S rDNA gene	GGGCGGTGTGTACAAGGC	(115)
16SF	466 bp of the 16S rDNA gene	TCCTACGGGAGGCAGCAGT	(141)
16SR	466 bp of the 16S rDNA gene	GGACTACCAGGGTATCTAATCCTGTT	(141)

Degenerate base code: N = G, A, C or T; D = G, A, or T; H = A, T, or C; K = G or T; M = A or C; R = A or G; S = C or G; V = G, A or C; W = A or T; Y = T or C; I = Inosine.

The cycle conditions varied for each PCR (but each was conducted for 50 cycles), and are detailed in following sections. Following PCR, an aliquot of each sample (10 µL) was analysed by agarose gel electrophoresis.

2.8.2.1 PCR of the human β-globin gene

PCR of the human β-globin gene was used to test the intactness of the purified template. This utilised the primers and method of Saiki (177) (Table 2.3) which targeted a range of amplicon sizes. The 1537r primer was novel to this study.

2.8.2.2 PCR of the *Y. enterocolitica* pYV gene *virF*

The pYV-located *virF* gene was amplified using the method and primers of Thisted Lambertz (194).

2.8.2.3 Gene-specific PCRS

The gene (*mecA*) was selected for detection of a methicillin resistant *S. aureus* with the primers and method of Barski (15).

The *Y. enterocolitica ail* gene PCR was performed using the primers and method of Wannet (208). This PCR had been used to detect *Y. enterocolitica* in food.

2.8.2.4 Genus-specific PCRs

The *tuf* PCR (for all staphylococcal spp.) which targets the staphylococcal elongation factor Tu (EF-Tu) used the primers and method of Martineau (127).

The yersinial target *ymoA* was selected for study with the primers and method of Grant (85).

2.8.2.5 Gram-positive and Gram-negative PCRs

The 16S Gram-positive and Gram-negative PCRs were both conducted using the method of Klausegger (114). They used a generic 16S primer (DG74), paired with a Gram-positive (143) or Gram-negative (68d) specific primer.

2.8.2.6 16S universal PCR - 333 bp

The 333bp 16S universal PCR was conducted with the primers DG74 and 68d and method of Greisen (88).

2.8.2.7 16S universal PCR - 1361 bp

The 16S Gram-positive and Gram-negative PCRs were both conducted in the method of Lane (117). All reagents and plasticware used for universal PCR were UV treated prior to the addition of template DNA.

2.8.3 Real-time PCR

Real-time PCR was conducted in a Rotor-Gene (Corbett Research), using SYBR-green chemistry. The gain was set to a value of 4 for each run, and the acquisition step varied depending on the PCR (see following sections). A melt profile was conducted at the end of the final extension. Template DNA was prepared as per Section 2.6.3. Five microlitres of template were used in each PCR. If necessary, an aliquot of PCR reaction (10 µL) was analysed by agarose gel electrophoresis. Real time results were analysed using RotorGene 4.6 software (Corbett Research).

2.8.3.1 Home-made SYBR green mix

The 20 µL PCR reaction also contained 2 µM (each) oligonucleotide primer, 1 ×PCR buffer (10 mM Tris-HCl, 50 mM KCl) (Applied Biosystems), 250 µM (each) dNTP, 1.2 to 2.4 mM $MgCl_2$ (Applied Biosystems), 1 U AmpliTaq GOLD DNA polymerase (Applied Biosystems) and SYBR green (supplied at 10 000× and diluted with ddH_20 to 1 in 30 000 for use, Molecular Probes).

2.8.3.2 Invitrogen Platinum SYBR Green qPCR SuperMix UDG

The PCR SuperMix contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP, 1 U UDG, 3 mM $MgCl_2$ (Applied Biosystems), 1.5 U Platinum *Taq* DNA polymerase and SYBR green, to which 10 µM (each) oligonucleotide primer was added. Three microlitres of template was added to each sample.

2.8.3.3 16S universal PCR

The method and primers of Nadkarni (143) were selected for detection of the 16S gene. Initially, a home-made mastermix (Section 2.8.3.1) was used, but then replaced in favour of a commercially available mastermix (Section 2.8.3.2). The cycles were increased to 50 to maximise the sensitivity of the PCR, and the gain was calibrated for each run (usually set to 6).

2.9 DNA sequencing

2.9.1 Preparation of DNA template

PCR product DNA was purified for sequencing using the Qiaquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions.

2.9.2 Sequencing reactions

Determination of nucleotide sequence was performed by cycle sequencing using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit Version 3, according to the manufacturer's directions (Applied Biosystems). PCR product (10 to 100 ng), was used as template in a 20 µL reaction with 4 µL of Terminator Ready Reaction Mix and 3.2 pmol (PCR template) oligonucleotide primer. Sequence reactions containing PCR product were subjected to 25 cycles of 96°C for 10 s (denaturation), 50°C for 5 s (annealing) and 60°C for 4 min (extension) using a GeneAmp PCR system 9700 (Applied Biosystems). Extension products were purified by ethanol precipitation. The 15 µL reaction mixture was combined with 25 µL distilled water, 4 µL of 3 M sodium acetate (pH 4.6), and 100 µL of 95% ethanol and placed on ice for 10 min. After centrifugation at 12 000 ×*g* for 30 min, the pellet was washed with 500 µL of 70% ethanol and dried at 80°C. Amplification products were run on an ABI 3730 automated capillary DNA sequencer (Applied Biosystems).

2.9.3 Analysis of sequence data

PCR products were sequenced in both directions, and submitted to the BLASTN Algorithm for analysis at The National Centre for Biotechnology Information. Sequence comparisons of the 16S rDNA gene between species were conducted when necessary using the CLUSTALW program.

2.10 Polymerase chain reaction – enzyme linked immuno assay

2.10.1 Biotinylated oligoprobes for PCR-ELISA

Probes used in the PCR-ELISA dig kit were purchased ready-made with a 5' biotin label from Proligo (Table 2.4). Oligoprobe sequences were obtained from published sources where indicated, or were designed using Primer3 software, following alignment and analysis of 16S sequence using CLUSTALW to find unique sequences for probing.

2.10.2 Digoxigenin-labelling of PCR products

Double-stranded DNA fragments (50 to 500 ng) were labelled with digoxigenin-11-dUTP (DIG) according to the manufacturer's instructions (Roche Applied Science). DIG-labelled DNA was stored at -20°C.

2.10.3 Hybridisation and washing conditions

PCR-ELISA was conducted using the Roche PCR-ELISA DIG-Detection kit Roche Applied Science), according to the manufacturer's protocol A instructions Briefly, in a 96-well plate DIG-labelled PCR product (usually 5 µL) was added to 20 µL Solution 1a (Denaturation solution, vial 1), which was then mixed and incubated for 10 min at RT. The volume of each sample was adjusted to 250 µL by adding the appropriate amount of solution 2a (Hybridisation solution with 7.5 pM/mL biotinylated probe), followed by vortexing. A 200 µL volume of each sample was pipetted into the MTP strips supplied with the detection kit (streptavidin coated). The plates were sealed with cover foils (supplied with the kit) and incubated at 55°C for 30 min with shaking.

Table 2.4: Biotinylated oligoprobes used in this study for PCR-ELISA

Name	16S Target	Sequence (5'-3')	Reference
Bcer	*B. cereus*	*GAACAAGTGCTAGTTGAATAAGC	This study
Coryne	*Corynebacterium* spp.	*GAAGCTTTTTGTGACGGTACC	This study
Enc131	*Enterococcus* spp.	*CCCCTTCTGATGGGCAGG	(17)
Enc145	*Enterococcus* spp.	*GGGATAACACTTGGAAAC	(17)
Enc1259	*Enterococcus* spp.	*GAAGTCGCGAGGCTAAGC	(17)
Ent	Enterobacter spp.	*CCCCCWCTTTGGTCTTGC	(106)
GBS	group B streptococcus	*GAACGTTGGTAGGAGTGG	This study
Koxy	*K. oxytoca*	*GAAGGGAGTGAGGTTAATAACC	This study
Paer	*P. aeruginosa*	*GAAGGGCAGTAAGTTAATACC	This study
Saga	group B streptococcus	*GTAAACACCAAACMTCAGCG	(195)
Sau	*S. aureus*	*GAAGCAAGCTTCTCGTCCG	(106)
Spn	*Strep. pneumoniae*	*GTGATGCAAGTGCACCTT	(106)
Spy	group A streptococcus	*TTCCAAAGCGTACATTGGTT	(195)
Str	*Streptococcus* spp.	*CACTCTCCCCTTCTGCAC	(195)

Degenerate base code: N = G, A, C or T; D = G, A, or T; H = A, T, or C; K = G or T; M = A or C; R = A or G; S = C or G; V = G, A or C; W = A or T; Y = T or C. * indicates biotin tag at 5' end.

The hybridisation solution was removed, and the plate washed five times with 250 µL solution 3a (washing solution, working solution) by aspiration. After the final washing, plates were tapped dry on lint-free cloths. A 200 µL volume of solution 4a (anti-DIG-POD working solution) was added to each well and the plate incubated for 30 min in the dark at 37°C with shaking. The anti-DIG-POD solution was removed, and the plate was again washed five times with 250 µL solution 3a by aspiration. After the final wash, plates were tapped dry on lint-free cloths. A 200 µL volume of ABTS solution (vial 6) was added to each well, and the plate incubated in the dark for 30 min with shaking at 37°C. Plates were read at 405 nm on a spectrophotometer (Biorad 3550). The results were then subtracted from the negative controls and plotted.

2.11 Southern dot blot

2.11.1 Design of oligoprobes

Oligoprobes used in this study were manufactured by Proligo and are listed in Table 2.5. Oligonucleotide sequences were obtained from published sources where indicated, or were designed using CLUSTALW alignments and BLASTN analysis of bacterial 16S gene sequences, and the Primer3 program.

2.11.2 Reverse-Southern dot blot

2.11.2.1 Digoxigenin-labelling of PCR products

Double-stranded DNA fragments (50 to 500 ng) were labelled with digoxigenin-11-dUTP (DIG) according to the manufacturer's instructions (Roche Applied Science). DIG-labelled DNA was stored at -20°C.

2.11.2.2 Poly dT tailing of oligonucleotides

Where necessary, oligonucleotide probes were poly dT tailed using the Roche DIG Oligonucleotide Tailing Kit (Roche Applied Science), with standard dNTPs (Roche Applied Science) instead of the supplied DIG-labelled dNTPs, according to the manufacturer's instructions.

Table 2.5: Oligoprobes used in this study for Southern dot blot

Name	16S Target	Sequence (5'-3')	Reference
143+3	Gram-positive bacteria	GGAYGACGTCAARTCMTCATGC	This study*
Bcer1	*B. cereus*	AAGAGCTGCAAGACCGCGAG	This study
Enc131	*Enterococcus* spp.	CCCCTTCTGATGGGCAGG	(17)
Entero1	Family Enterobacteriaceae	CTCGCGAGAGCAAGCGGAC	This study
N6R	Gram-negative bacteria	GGTGCCTTCGGGAAC	(42)
Paer1	*P. aeruginosa*	GCCTTGACATGCTGAGAACTTTCC	This study
Ppunew5r	*Pseudomonas* genus	GGGTTGCCAAGCCGCGTG	This study
Saga	group B streptococcus	GTAAACACCAAACMTCAGCG	(195)
Saur2	*S. aureus*	GAAGCAAGCTTCTCGTCC	(106)
SP16SR	Universal 16S	CTACGCATTTCACCGCTACAC	(90)
Spy3	group A streptococcus	TTCCAAAGCGTACATTGGTTGAGC	This study
Staph3	*Staphylococcus* genus	CTTACACATTTGTTCTTCCCTAATAACAG	This study
STREP16SR	*Strep. mitis/ Strep. pneumoniae*	GTACCGTCACAGTATGAACTTTCC	(51)
Yent2	*Y. enterocolitica*	ACTCTTGACATCCACGGAATTTAGC	This study

Degenerate base code: N = G, A, C or T; D = G, A, or T; H = A, T, or C; K = G or T; M = A or C; R = A or G; S = C or G; V = G, A or C; W = A or T; Y = T or C.
* Derived from oligonucleotide 143 (see Table 2.3).

2.11.2.3 Binding of oligoprobes to the nylon filters

One microlitre aliquots of appropriately diluted (between 0.2 and 50 pM) oligonucleotide probes were spotted in duplicate onto Hybond-N+ nylon membrane (Amersham Biosciences). The oligoprobes were fixed to the membrane with a Spectrolinker XL-1000 UV crosslinker (Spectronics Corporation).

2.11.2.4 Probing of nylon filters

The DIG-labelled PCR products were added to DIG Easy Hyb Buffer (Roche Applied Science) and denatured by boiling. Prehybridisation was performed for at least 2 h at between 40 and 60°C (depending on the experiment). Membranes were hybridized overnight with the DIG-labelled PCR product and with gentle agitation at the same temperature selected for pre-hybridisation. The membranes were then washed twice with 2 ×SSC, 0.1% (wt/vol) SDS for 5 min at RT, followed by 2 washes with TMAC buffer (3 M tetramethylammoniumchloride, (TMAC), 50 mM TrisCl pH 8, 0.2 mM EDTA, 0.1% wt/vol SDS) at 40°C for 15 min. Blocking solution was added and the membrane incubated at RT for 30 min.

2.11.2.5 DIG detection

After blocking, hybridised DIG-labelled PCR product was detected with alkaline phosphatase conjugated anti-digoxigenin antibody, and 5 to 15 min after the addition of the chemiluminescent alkaline phosphatase substrate, CDP-Star (Roche Applied Science), membranes were exposed to Kodak BioMax Light Film. Films were developed using an automated X-ray film developer (Kodak).

2.11.3 Standard-Southern dot blot

2.11.3.1 3' Digoxigenin end labelling of oligonucleotides

Oligonucleotide probes were DIG-labelled using the Roche DIG Oligonucleotide Tailing Kit (Roche Applied Science), according to the manufacturer's instructions.

2.11.3.2 Binding of PCR products to the nylon filters

One microlitre aliquots (50 to 100 µg of template) of PCR product were spotted in duplicate onto Hybond-N+ nylon membrane (Amersham Biosciences). These were fixed to the membrane with a Spectrolinker XL-1000 UV crosslinker (Spectronics Corporation). Where necessary, these PCR products were purified with the Qiagen QIAquick PCR Purification Kit (Section 2.9.1) prior to their application to the nylon membrane.

2.11.3.3 Probing of nylon filters

The DIG-tailed oligoprobes were each added to DIG Easy Hyb Buffer (Roche Applied Science) at a concentration of 10 pM/µL. Prehybridisation was performed for at least 2 hours at 50°C. Membranes were hybridised overnight with the DIG-labelled oligoprobe and with gentle agitation at 50°C. The membranes were then washed twice with 2 ×SSC, 0.1% (wt/vol) SDS for 5 min at RT, followed by 2 washes with 0.1 ×SSC, 0.1% (wt/vol) SDS at 50°C for 15 min. Blocking solution was added and the membrane incubated at RT for 30 min.

2.11.3.4 DIG detection

After blocking, hybridised DIG-labelled oligoprobe was detected with alkaline phosphatase conjugated anti-digoxigenin antibody, and 5 to 15 min after the addition of the chemiluminescent alkaline phosphatase substrate, CDP-Star (Roche Applied Science), membranes were exposed to Kodak BioMax Light Film. Films were developed using an automated X-ray film developer (Kodak).

CHAPTER THREE

SURVIVAL AND PROLIFERATION OF BACTERIAL CONTAMINANTS IN BLOOD COMPONENTS PRODUCED BY THE BUFFY COAT METHOD

3.1 Introduction

Prospective screening has revealed a diverse range of bacteria contaminating blood products (14, 20, 26, 65, 116, 119, 128, 174, 175, 225). Although they are generally introduced into the products at low numbers (7, 24, 57, 76, 78, 201), little is known about their behaviour in these during storage, or how long it takes them to reach numbers of risk to recipients. Additionally, not all bacteria identified during screening have been associated with patient sepsis, and it has not been determined whether cases involving these species have simply failed to be recognised, or whether they are unable to proliferate to significant numbers during product storage. Some studies of bacteria spiked into products manufactured by the platelet rich plasma (PRP) method have been published (160, 199), but these products are not widely used in Australia and differ significantly from the those which are, a process known as the buffy coat method. A review of these products can be found in Section 1.2.3, but most importantly, buffy coat products contain fewer WBCs and little natural plasma. Little is known about what effects the current practices of reducing WBCs and plasma (hence complement) in products, has on the ability of bacteria to grow and survive, although it is hypothesised that buffy coat products may have a lesser ability to prevent bacterial outgrowth. We therefore wished to:

1. Determine the behaviour of bacteria deliberately spiked into buffy coat PCs and RCCs;
2. Evaluate if the bacteria can survive and grow in these storage environments;
3. Determine how few microorganisms are required for detectable bacterial outgrowth; and
4. Determine which bacteria are of risk to patients (and hence worth screening for by means of a rapid detection method).

To this end, growth kinetic studies of bacteria in stored buffy coat blood products were undertaken.

3.2 Selection of bacteria for study

It was impractical to study all contaminant species in blood products for their growth kinetics. Subsequently, published (164, 200) and ARCBS survey data were used to categorise each into four main groups, and representatives of each category were selected for study. The groups identified and bacteria chosen to represent them were: psychrophilic, environmental opportunist (*P. fluorescens*); mesophilic, environmental opportunist (*P. aeruginosa*); psychrophilic enteric (*Y. enterocolitica* O3); and skin flora (*S. aureus* and *S. epidermidis*). All of these species have been reported as causing transfusion-associated fatalities, and where possible, isolates from such cases were used (116, 136, 182, 200) *Y. enterocolitica* serotype O3 was chosen for study, as this serotype has been most commonly implicated in *Y. enterocolitica* transfusion incidents in Australia and New Zealand (68, 109, 123). The presence pYV in the *Y. enterocolitica* strain 67R was confirmed by PCR at the time of spiking (not shown). The identity of all isolates was confirmed by using a VITEK system.

Fresh bacterial cultures and the blood products were prepared as per Sections 2.5.1 and 2.5.2. PCs contained 8×10^7 WBCs/pack, whilst RCCs contained $\sim 10^8$ WBCs/pack.

3.3 Platelet concentrates

The growth curves of *P. fluorescens*, *P. aeruginosa*, *S. epidermidis*, *S. aureus*, and *Y. enterocolitica* in PCs are shown in Figure 3.1. The actual bacterial inocula for the 10^2 CFU/mL experiments were 115, 108, 35, 130, and 334 CFU/mL, for *P. aeruginosa*, *P. fluorescens*, *S. aureus*, *S. epidermidis*, and *Y. enterocolitica*, respectively. The actual bacterial inocula for the 10 CFU/mL experiments were 13, 2, 8, 17, and 37 CFU/mL, *for P. aeruginosa*, *P. fluorescens*, *S. aureus*, *S. epidermidis*, and *Y. enterocolitica*, respectively. As few as 5 CFU/mL of these bacteria grew to $>10^5$ CFU/mL in stored PCs within 3 d of spiking.

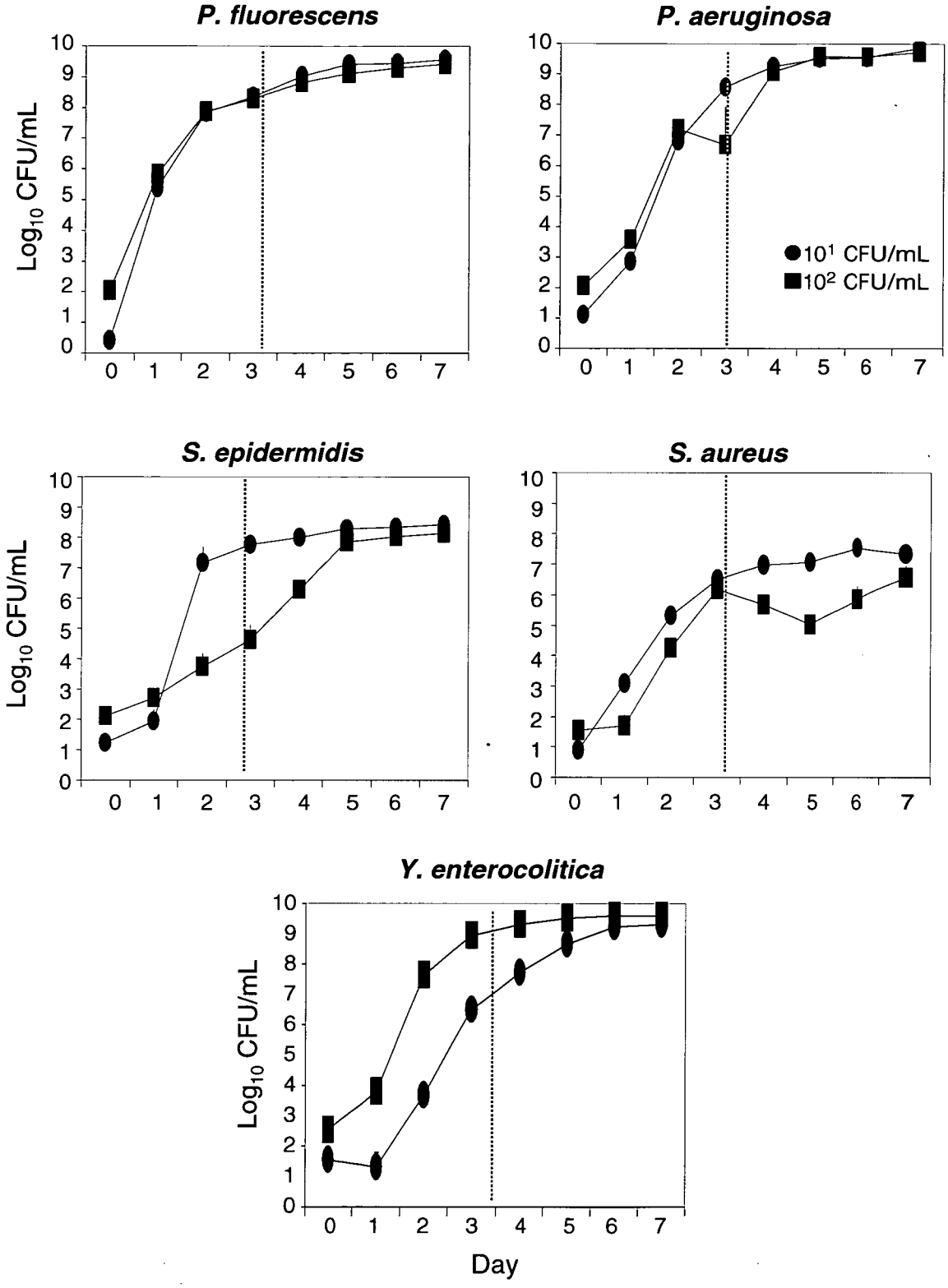


Figure 3.1: Bacterial growth in stored (22±2°C, 7 d) platelet concentrates

Vertical lines indicate when product spoilage (loss of swirling, platelet aggregates or product cloudiness) became evident.

P. fluorescens displayed logarithmic growth soon after spiking, and peaked at 10^8 CFU/mL within 2 d. *P. aeruginosa* displayed similar, although slightly slower growth, achieving 10^8 CFU/mL in 2.5 d. *S. aureus* and *S. epidermidis* proliferated more slowly than the pseudomonads, and achieved 10^7 CFU/mL by 3 d. The *Y. enterocolitica* strain 67R commenced logarithmic growth after 24 h. All bacteria that proliferated achieved maximal numbers at around 4 d. Gram-negative bacteria peaked at approximately 10^9 CFU/mL, whereas *S. aureus* and *S. epidermidis* achieved were between 10^7 and 10^8 CFU/mL. The growth curves obtained for each species at both inocula tested were similar.

3.3.1 Spoilage of platelet concentrates

All species that proliferated caused spoilage of PCs (indicated by the vertical dotted lines), but this was not evident until after 3 d when the Gram-positive bacteria had reached 10^6 CFU/mL, and the Gram-negative bacteria were around 10^8 CFU/mL. Of the bacteria tested, only the *Staphylococcus* spp. induced the formation of platelet aggregates in PCs. For *S. epidermidis*, numerous aggregates of 3 mm diameter were seen (not shown). For *S. aureus*, aggregates consisted of one or two large clumps of 10 to 40 mm in diameter (Fig 3.2b). In PCs containing the other bacterial species, an increase in the 'cloudiness' of the pack was observed (Figure 3.2c). A lack of PC swirling was evident in all highly contaminated PCs, regardless of the other indicators of spoilage observed. Absence of swirling was observed in some control PCs at 7 d, although sampling revealed that these remained sterile.

3.4 Red cell concentrates

The actual bacterial inocula for the 10^3 CFU/mL experiments were, 481, 906, and 1360 CFU/mL for *P. aeruginosa*, *S. aureus* and *S. epidermidis*, respectively. The starting inocula for the 10^2 CFU/mL experiments were 69, 113, 449, 34, and 377 CFU/mL for *P. aeruginosa, P. fluorescens*, *S. aureus*, *S. epidermidis,* and *Y. enterocolitica*, respectively, whilst the starting for the 10 CFU/mL experiment were 7, and 32 CFU/mL for *P. fluorescens* and *Y. enterocolitica*, respectively (Figure 3.3) All groups of bacteria were recovered from the spiked RCCs after 36 d of storage.

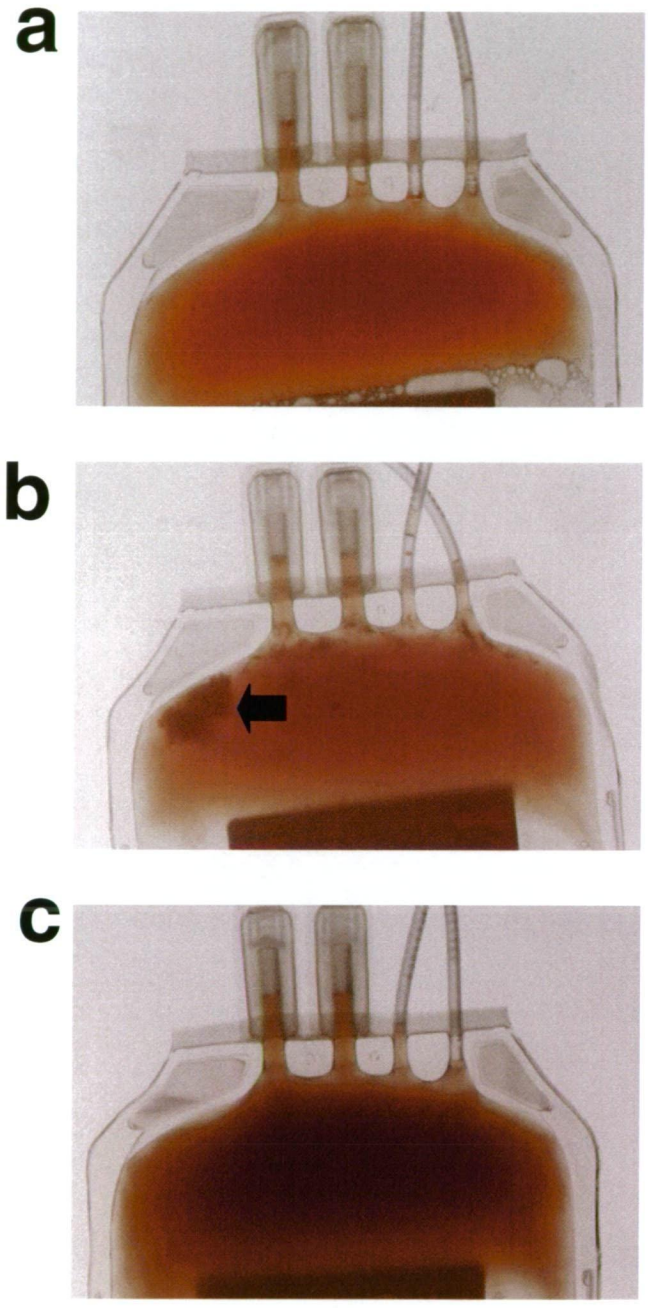


Figure 3.2: Spoilage markers of platelet concentrates

a) Control PC.
b) Contaminated PC containing ~10^8 CFU/mL *S. aureus*.
The arrow indicates aggregate of ~ 3 cm diameter.
c) Contaminated PC containing ~10^8 CFU/mL
P. aeruginosa, showing product cloudiness.
NB: swirling was unable to be photographed clearly.

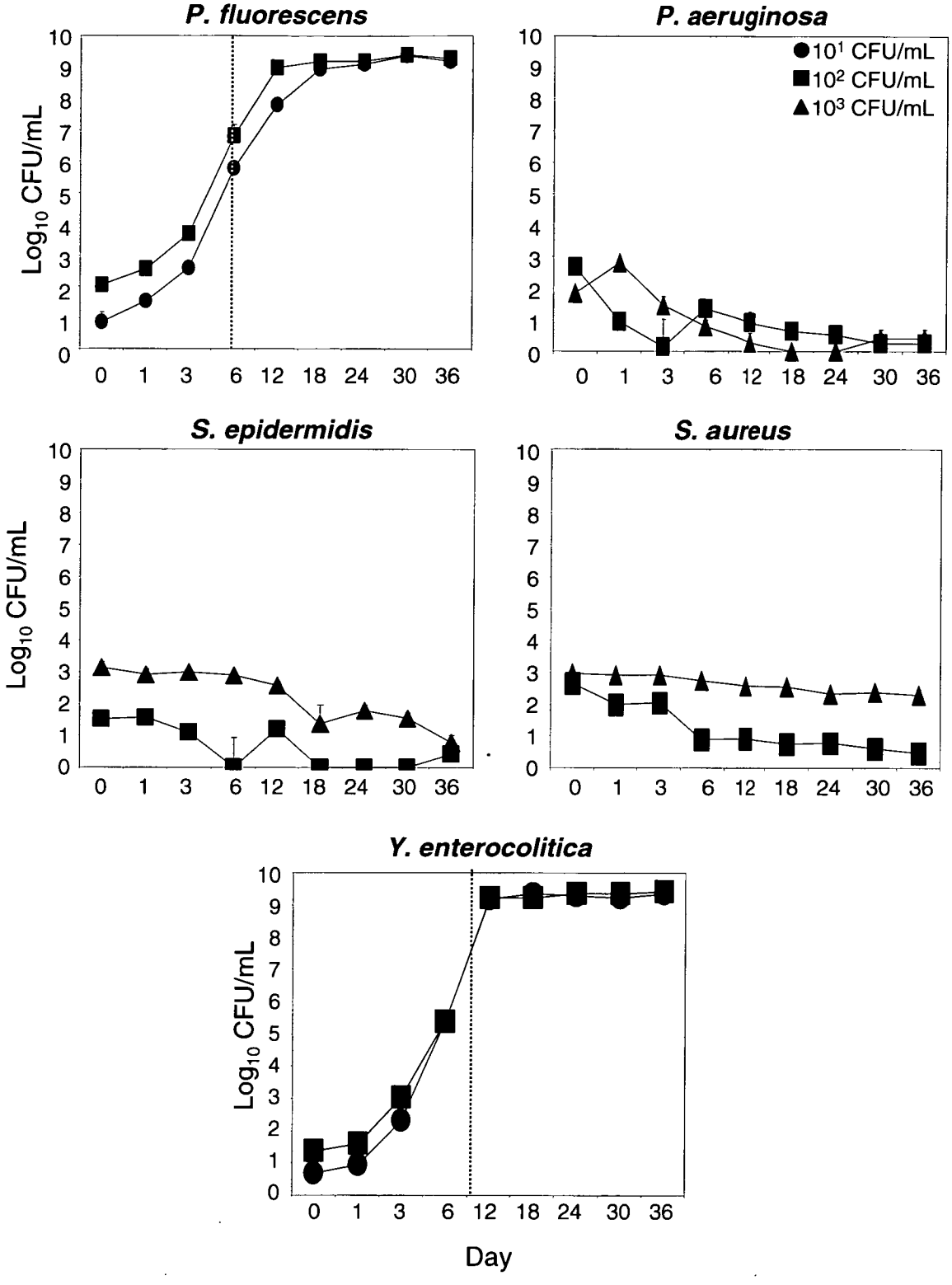


Figure 3.3: Survival and growth of bacteria in stored (4±2°C, 36 d) red cell concentrates

Vertical lines indicate when product spoilage (pack darkening due to red cell haemolysis) became evident.

However, only the psychrophilic species (*Y. enterocolitica* and *P. fluorescens*) proliferated, with both microorganisms reaching stationary phase by around 12 d. Fewer than 10 CFU/mL of *Y. enterocolitica* and *P. fluorescens* were able to initiate growth which began after a 24 h lag period. In contrast, the numbers of *P. aeruginosa, S. aureus* and *S. epidermidis* declined within the first 6 d of spiking. As a result, these bacteria were not recovered on several occasions during the early period of experimentation, but were isolated at later time-points in low numbers (approximately 5 CFU/mL), indicating they survived extended product storage under standard conditions. The proliferative patterns of the psychrophiles did not appear to be influenced by the size of the inoculum. However, the Gram-positive organisms appeared to survive slightly more efficiently (10^2 to 10^3 CFU/mL) when spiked at the higher 10^3 CFU/mL dose, compared with the lower 10^2 CFU/mL dose (survival at 5 to 10 CFU/mL). *P. aeruginosa* survival was unaffected by the size of the inoculum.

3.4.1 Spoilage of red cell concentrates

There was no evidence of spoilage in products spiked with the species that survived, but did not proliferate (Figure 3.4a). The actively proliferating species did, however, cause the characteristic pack darkening indicative of spoilage (Figure 3.4b). For *P. fluorescens*, spoilage of RCCs (indicated by the vertical dotted lines on the growth plots) was evident at 6 d (10^7 CFU/mL), and for *Y. enterocolitica* at 9 d (10^7 and 10^8 CFU/mL).

3.5 Y. enterocolitica

Despite reports linking TTBS to *Y. enterocolitica*-contaminated PCs, this species had previously been reported as unable to proliferate in these products. Therefore, we wished to determine the contribution of the anti-host 70 kb virulence plasmid, pYV, to survival and growth of this species in stored buffy coat PCs, as this plasmid may be lost during growth of bacterial cultures for spiking (which may have occurred in previous studies).

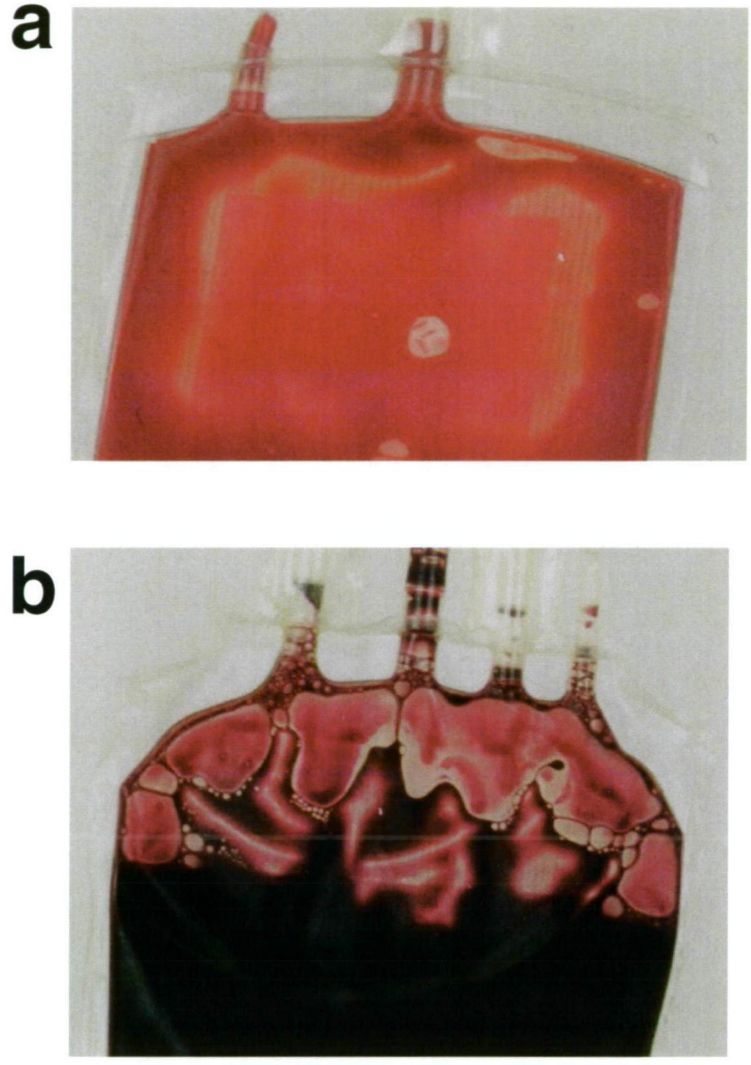


Figure 3.4: Spoilage markers of red cell concentrates

a) Control RCC.

b) Contaminated RCC containing ~10^8 CFU/mL *P. fluorescens.* Note the extensive haemolysis and pack darkening.

We also wished to evaluate the role of pYV in the survival and growth of *Y. enterocolitica* in RCCS. To this end, a virulence plasmid-less (67W) variant of *Y. enterocolitica* O3 strain 67 was subsequently studied in stored buffy coat PCs and RCCs, and its behaviour compared with that of the parental strain.

3.5.1 Platelet concentrates

The actual spiking numbers of *Y. enterocolitica* strain 67W were 45 and 3 for the 10^2 CFU/mL and the 10 CFU/mL experiments, respectively. The pYV+ *Y. enterocolitica* strain 67R commenced logarithmic growth after 24 hours, whereas the pYV- *Y. enterocolitica* strain 67W was unrecoverable after 3 d (Figure 3.5a). Spoilage was only evident in the 67R-spiked PCs at 3 to 4 d (10^8 CFU/mL)

3.5.2 Red cell concentrates

The actual spiking numbers of *Y. enterocolitica* strain 67W were 40 and 8 for the 10^2 CFU/mL and the 10 CFU/mL experiments, respectively. The growth curves of both strains of *Y. enterocolitica* were identical (Figure 3.5b). Presence or absence of pYV in the *Y. enterocolitica* strains did not appear to affect the time-point at which spoilage occurred, nor the number of bacteria required to cause it.

3.6 Discussion

The bacterial behavior observed in stored buffy coat PCs suggests that these products have reduced antimicrobial activity, compared to plasma-rich PCs. Not only did some bacterial contaminants survive throughout blood product storage, but some species also proliferated to reach high numbers without producing physical evidence of contamination. The introduction of even small numbers of bacterial contaminants (less than 10 CFU/mL) into buffy coat PCs and RCCs may provide an opportunity for bacterial outgrowth. In buffy coat PCs, we observed maximum counts of the Gram-positive species 1 to 2 d earlier, and the Gram-negative species 1 day earlier (for 10^8 CFU/mL) than counts reported in PRP PCs by Punsalang (158).

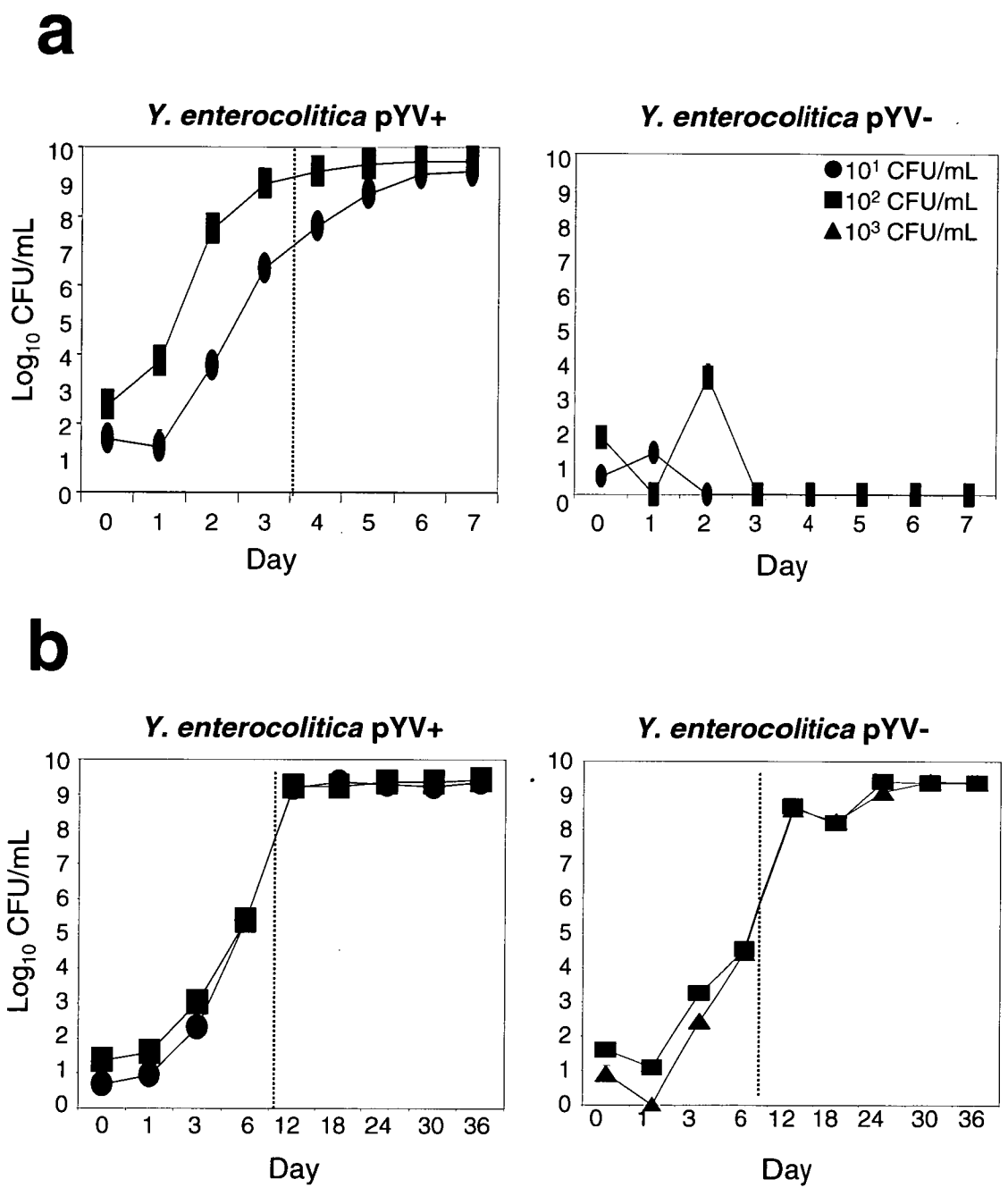


Figure 3.5: Survival and growth of pYV+ and pYV- *Y. enterocolitica* strains in blood products

a) PCs

b) RCCs

Vertical lines indicate when product spoilage became evident.

Likewise, the proliferation of *S. epidermidis* and *S. aureus* (with inocula of 7 and 85 CFU/mL, respectively) to stationary phase in PRP PCs in a study by Braine (31) was slower than in the buffy coat PCs used in this study. However, it is possible that strain variation may be partially responsible for these observations, as other studies using the same species of bacteria have shown similar results to ours (46, 201). As the average age of transfused PCs is estimated to be 3 to 4 days old, it is concerning that contaminated products may have already reached significant clinical levels by this stage, although they do not show the physical indicators of extreme contamination. Certainly, many instances of TTBI and sepsis have been recorded following transfusion of PCs of this age, and with no apparent indicators of contamination. It is, therefore, imperative that another means of identifying these contaminated products and removing them from the supply is developed.

A consistent finding of our study of spiked buffy coat-reduced RCCs was that the bacteria within these remained viable for at least 36 d. Bacteria which grew at low temperatures proliferated rapidly to high numbers, whereas other species persisted in low numbers. Whilst highly contaminated RCCs pose obvious risks, there is increasing clinical evidence that lower numbers of bacteria may also be of concern, particularly in immunocompromised patients. Although most TTBI/deaths from bacterially contaminated RCCs are associated with rapidly growing psychrophiles (200), other bacteria, such as *S. aureus* and *S. epidermidis*, have also been implicated in patient deaths (116, 164, 216, 217). In these instances, the presence of superantigens (140) or other bacterial toxins may play a role in the pathogenesis of the disease. Alternatively, the combination of a small number of live bacteria and the remnants of dead bacteria (such as occurs in a patient undergoing antibiotic therapy and who subsequently receives a contaminated product) may be sufficient to exert the same effect. Cytokines produced during product storage may also be important in transfusion-related sepsis, but the low number of WBCs contained within buffy coat-reduced RCCs, and the low temperature conditions of storage are likely to limit this.

In contrast to our results, other published studies have failed to show proliferation of *Y. enterocolitica* in stored PCs (38, 201). We have demonstrated in repeated experiments that survival and growth of *Y. enterocolitica* in PCs requires

the virulence plasmid, pYV, which confers serum resistance and resistance to phagocytosis. It also appears that despite the reduced amount of WBCs and complement in these products, the plasmid-less *Y. enterocolitica* strain 67W remained sensitive to killing. Therefore, despite the reduced presence of antibacterial mechanisms in PCs, bacterial species and strains classified as sensitive to serum and/or phagocytic killing may still be cleared from these products. Plasmid-less *Y. enterocolitica* strains readily occur spontaneously in vitro, particularly when the bacteria are cultivated at 37°C. Hence, it seems likely that other investigators may have grown pYV- strains of this species, although there is also the possibility some variation in survival between O-types (serotype O8 was more frequently studied elsewhere). Regardless of the mechanism of bacterial survival, the importance of *Y. enterocolitica* contamination of stored PCs was emphasised by reports of PC-related fatalities attributable to *Y. enterocolitica* sepsis (55, 97, 109, 116, 164, 181, 200).

Although pYV was required for *Y. enterocolitica* survival in PCs, this was not the case in stored buffy coat RCCs. Indeed, the similar growth curves of the pYV+ and pYV- strains in RCCs established that pYV does not influence the survival or proliferation of this species in buffy coat RCCs. The non-requirement of pYV for the survival of *Y. enterocolitica* in RCCs can be explained by the low levels of WBCs and plasma, and/or by reduced antimicrobial activity during refrigeration. Although pYV- bacteria are ill-equipped to survive in the recipients of contaminated blood, the amount of endotoxin produced during storage still poses a significant hazard (7, 134, 136).

There is, therefore, a need for a rapid and more sensitive screening technique to ensure a blood supply free from bacterial contamination. With the data available from this and previous studies, all contaminants should be considered of potential risk in one or more products, and should therefore be screened universally. Methods by which this might be achieved, were therefore evaluated.

CHAPTER FOUR

DEVELOPMENT AND EVALUATION OF RAPID METHODS FOR THE DETECTION OF BACTERIAL CONTAMINANTS IN BLOOD PRODUCTS

4.1 Introduction

More than 35 species of bacteria have to date been found to contaminate blood products (14, 20, 26, 65, 116, 119, 128, 174, 175, 225). The findings of Chapter 3 suggest that ideally, all bacteria should be detected and the product removed from the blood supply. It also showed that 10 CFU/mL may quickly proliferate to reach high numbers (>10^5 CFU/mL) in buffy coat products in as little as 2 to 3 d for PCs, and less than 12 d for RCCs, without being identifiable by visual inspection. For these reasons, contamination must be detectable at less than 10^3 CFU/mL, well before there is a significant risk to patients. As few bacteria are present during WB collection, detection is likely to be more successful if it occurs close to the time of transfusion.

Molecular methods such as PCR are increasingly being used to detect the presence of bacteria in a range of settings, from specific targeting of species, to generic amplification of all bacteria using conserved 16S ribosomal sequences (104, 176). Blood-banking institutions have recognised the potential of molecular methods by implementing nucleic acid testing (NAT) to complement their serological screening procedures for viruses. For these reasons, the molecular method of 16S PCR was selected for further investigation in this study. *Yersinia enterocolitica* (ATCC 23715) and *Staphylococcus aureus* (ATCC 25923) were chosen for initial evaluation of the detection method. Both are clinically significant contaminants of blood products, were able to survive and/or proliferate in the kinetic experiments in this study (Chapter 3), and allowed us to test the efficacy of our extraction and detection methods on both Gram-positive and Gram-negative species. After the initial establishment of our methods with these species, the range of bacteria was expanded to more then 30 species and strains found in blood products (Table 2.2).

4.2 Selection and development of a DNA extraction method

The screening of blood products for bacteria requires a high-throughput, rapid extraction method that can be conducted as simply and safely as possible. Furthermore, to ensure suitable sensitivity of the PCR assay, we need to effectively extract the nucleic acid from as many bacteria as possible from each sample.

Initially we examined the possibility of using commercial kits to extract DNA, as many such kits are available to obtain bacterial DNA from various sample types. The desired sensitivity of the PCR was set to 10^3 CFU/mL. If a 10 µL aliquot of prepared 100 µL template (from an original 1000 µL sample) is used, this equates to 100 CFU/reaction.

4.2.1 Qiagen QIAmp DNA Mini Kit

The Qiagen DNA Mini Kit is recommended for extracting DNA from both prokaryotic and eukaryotic cells, particularly in the presence of blood and blood products. The method for extraction of bacterial DNA involves several additional steps, as the bacterial cell wall is more difficult to lyse than the eukaryotic cell membrane. As the objective was to obtain bacterial DNA from both Gram-negative and Gram-positive species, the manufacturer's instructions for Gram-positive bacteria were used as these are more resistant to lysis (also see Chapter 2, Section 2.6.1). Initially, the method was evaluated using pure cultures, and then tested against spiked PCs and RCCs. Twenty microlitre pellets of starting material were used, and of the 100 µL eluted template, 10 µL was examined by electrophoresis (Figure 4.1a). Unfortunately, whilst some template DNA was recovered from *S. aureus*, the amount was much less than the amount obtained from *Y. enterocolitica*. Extension of the lysis buffer step to an overnight incubation, and varying the amount of lysozyme and lysostaphin in each reaction, did not enhance the yield (results not shown). Furthermore, the requirement of an overnight incubation step significantly slowed the extraction process, and our preferred sample volume of 1000 µL was unable to be processed, due to repeated clogging of the spin filter membranes.

Hence, it was apparent that this kit would not meet our needs, and a mechanical lysis method was investigated instead.

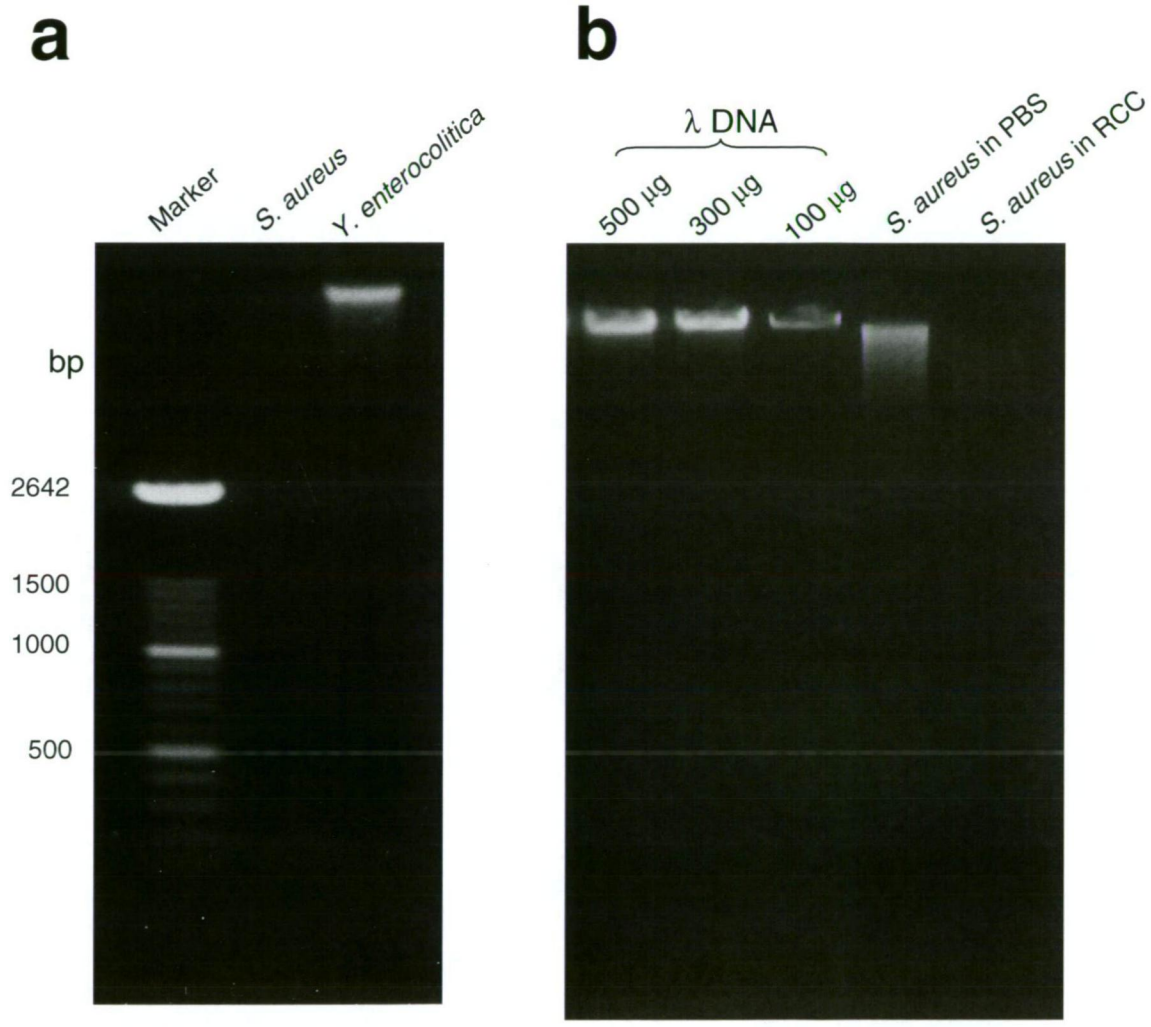


Figure 4.1: Kit extraction of bacterial DNA from washed cells

The source of DNA is shown above each lane.

a) Qiagen Mini Kit Protocol D for Gram-positive bacteria.

b) FastDNA SPIN Kit for Soil.

4.2.2 BIO 101 FastDNA SPIN Kit for soil

'Bead-beating' using tubes containing ceramic or glass beads or garnet powder, facilitates the release of DNA using ballistic disintegration of the cells in a milling machine. This has been shown to rapidly lyse a majority of cell types, and many samples can be processed simultaneously (29, 224). The BIO101 FastDNA SPIN Kit with the FastPrep bead-beating machine was chosen for evaluation. These have been successfully applied to soil samples containing bacteria, which like our blood products, are rich in particulate matter. This kit was used in accordance with the manufacturer's instructions (as detailed in the Materials and Methods chapter, Section 2.6.2), which are briefly described here.

Approximately 20 µL pellets of pure culture were processed (or known quantities of bacteria spiked into RCC or PC), and 10 µL of 100 µL of template eluent was examined by gel electrophoresis. As shown in Figure 4.1b, this kit successfully produced more DNA from pure cultures than the Qiagen kit, but just half of our target volume of 1000 µL of blood product could be processed in this way. Furthermore, the cellular debris from the RCCs caused the spin column membrane to repeatedly clog and rupture (hence little DNA recovered; Figure 4.1b). The red colour of the recovered 'purified' DNA from the RCC sample also indicated that this was significantly contaminated with haemoglobin (not shown). Repeated attempts to modify this method resulted in the same unsatisfactory results with spiked RCCs.

4.2.3 A novel rapid method for the extraction of DNA from bacteria in blood products

Although both Qiagen and the Bio101 kits met some our needs in terms of sample types and speed, neither was capable of extracting DNA from all bacteria and all blood products from a 1000 µL starting volume. Therefore, bead beating (for effective and rapid disruption of Gram-positive species, without the incubation period needed for enzymatic lysis) and spin column cleanup were combined. To process a 1000 µL sample, we added a pre-bead beating lysis step to remove the bulk of the cells from each blood product, particularly the RCCs.

The detailed protocol is provided in Chapter 2, Section 2.6.3. Briefly, up to a 50 µL pellet of washed bacteria, or a 1000 µL sample of spiked blood product was treated with pre-lysis buffer, mixed, and the debris was pelleted by centrifugation. After removal of the supernatant, the pellet was resuspended in the bead-beating lysis buffers, and treated in the FastPrep machine for 3 min on speed 5.5 to ensure maximum bacterial lysis. Potassium acetate was added to the recovered supernatant to precipitate protein in the sample, followed by Proteinase K treatment for any remaining protein. From this step, the Qiagen method of spin columns and wash buffers was used to clean up the samples and precipitate the DNA, prior to storage at 4°C in Buffer AE. Samples of our DNA extracts from 100 µg of washed bacterial cells (10 µL of 100 µL of total purified template, giving 200 µg/uL template) were examined by electrophoresis, and are shown in Figure 4.2. This extraction technique was highly successful with the target of 1000 µL of spiked blood product, and with all species of bacteria (>30) tested (results not shown). Some samples contained residual RNA, which was removed (when desired) by treatment with RNase.

Although some shearing of the template was evident by smearing of samples on gel electrophoresis, the majority of the sample appeared to be intact. To confirm the intactness of the template and minimise the risk of false-negatives (which would occur if the only intact template were smaller than our target), sterile PC and RCC controls were extracted and subjected to PCR for the β-globin gene using a range of primers for different length targets (Figure 4.3). All primer pairs produced amplicons, including the largest target of almost 1500 bp. These results showed that our template is both sufficiently intact and free from reaction inhibitors (such as haeme) and indicated that false negative reactions are unlikely to occur.

Having developed a method to obtain PCR quality DNA, we investigated various detection methods for bacteria in blood products.

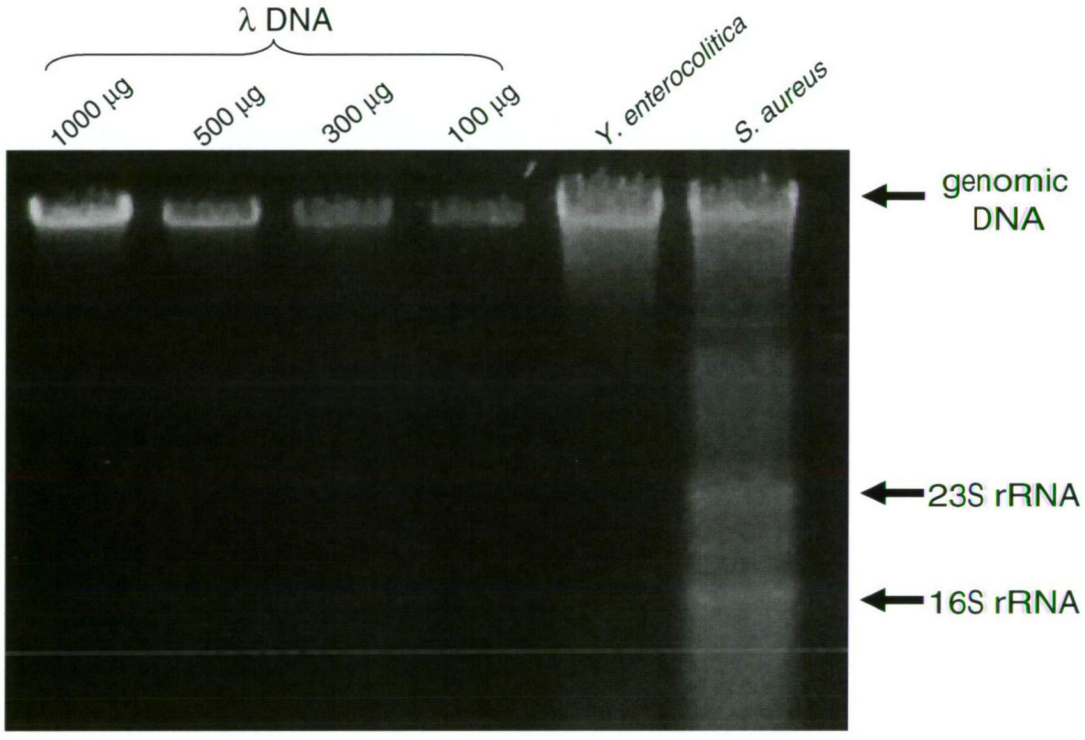


Figure 4.2: Extraction of DNA washed bacterial cells, using the developed novel rapid method of bead beating with spin column purification

The source of DNA is shown above each lane.

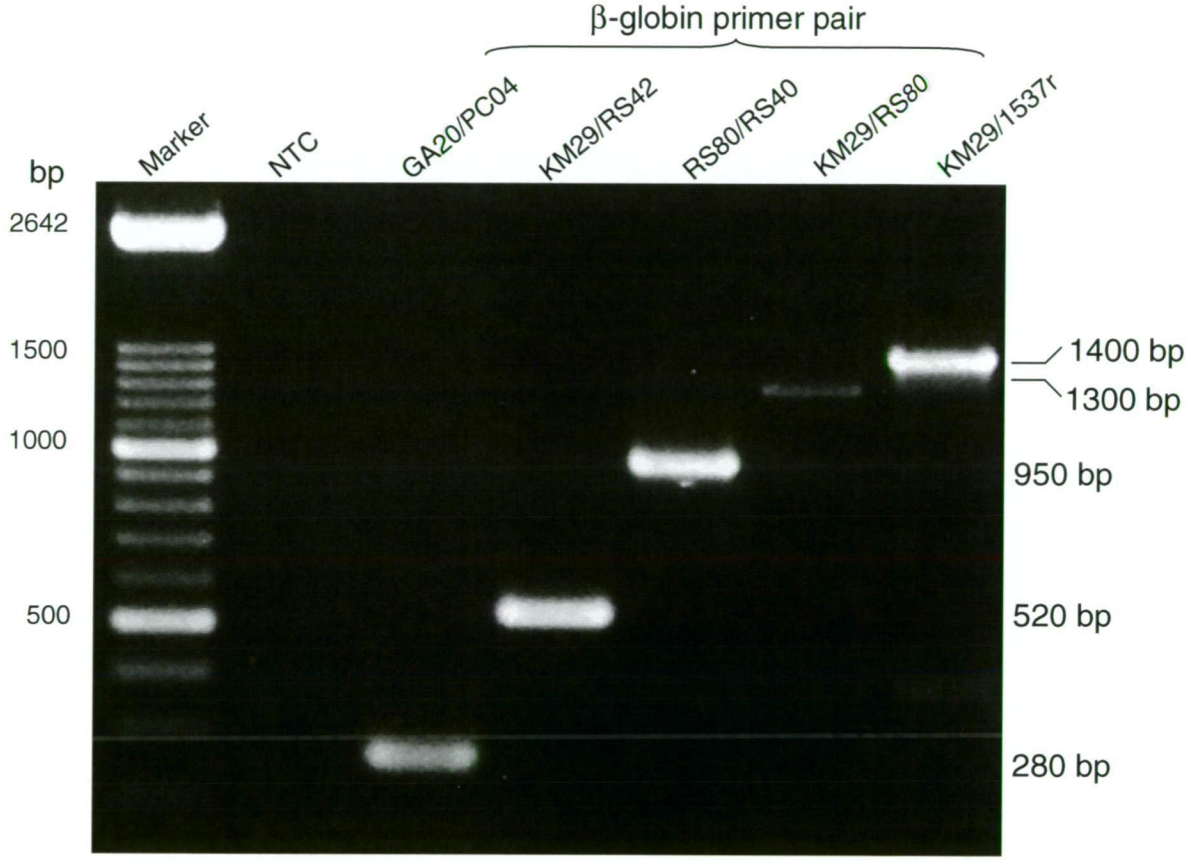


Figure 4.3: β-globin PCR demonstrating intactness and purity of DNA extracted from sterile PCs by our novel rapid method

NTC; no template control.

4.3 Detection of bacterial contaminants using PCR

As stated earlier, our ultimate goal was to use conserved 16S sequences to detect all bacterial contaminants in a single reaction. However, many factors may influence the ability to detect a target in any given sample. For a PCR reaction to be positive, the selected primers must be able to bind the target sequence, and the *Taq* polymerase must be able to use these to produce multiple copies. The presence of inhibitors (through poor template preparation), incorrect cycling conditions or design of the primers, may lead to false-negative results. These issues also apply to 16S universal PCR, but in addition, false-positive results may occur in sterile samples, due to the ubiquitous nature of the 16S template in the environment, and the conserved sequences which are being targeted (163). As indicated in Section 4.2.3, the template obtained was of PCR quality, but in order to address these other issues adequately and ensure no loss of specificity or sensitivity with universal PCR as compared to species-specific targeting, the approach adopted was: to sequentially detect *S. aureus* and *Y. enterocolitica* at the species, genus, Gram, and universal levels.

4.3.1 Bottom-up detection of *S. aureus* and *Y. enterocolitica*: species specific to universal 16S PCR

Figure 4.4 shows the genetic targets and the desired levels for testing the PCR process with our samples. These were selected as a majority of these assays were available from published methods. Comparisons of both standard and real-time PCR methodologies were adopted to ascertain which might be more suitable, as each has particular advantages (discussed in Chapter 1, Sections 1.8.1 and 1.8.2). The same gene targets were used for both methodologies.

4.3.2 Standard PCR amplification

Standard PCR amplification requires DNA polymerase and specific primers to manufacture many copies of a target which may be up to 30 kb or more in length, depending on the enzyme used. Post-cycling, samples are subjected to gel electrophoresis, where a presumptive identification of a positive reaction is made,

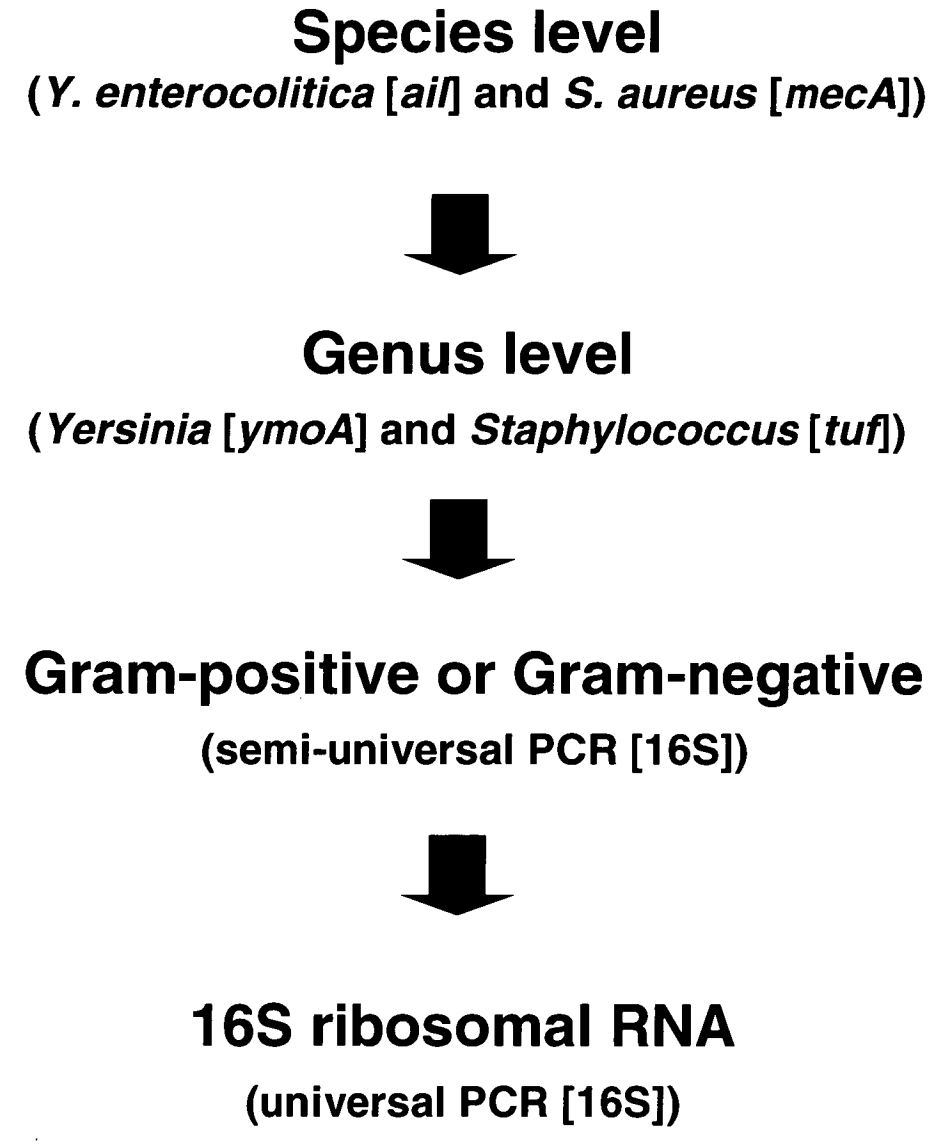


Figure 4.4: Strategy used to develop a method to detect bacteria using 16S universal PCR

based on the size of the amplified fragment. However, absolute confirmation of the identity of the PCR product must be made through sequencing or probing with known sequences (such as Southern dot blotting, discussed in Section 4.4.2).

4.3.2.1 Species-specific targets – *S. aureus mecA* and *Y. enterocolitica ail* genes

Our selected targets for species-specific identification of *S. aureus* and *Y. enterocolitica*, were *mecA* and *ail*, respectively, which have been successfully used with a variety of sample types such as milk and food (15, 208). A 10 µL volume of template was used in each reaction, as this was shown to increase the sensitivity of these PCRs. Typical results for the *S. aureus mecA* and *Y. enterocolitica ail* PCRs are shown in Figures 4.5a and 4.5b, respectively. A summary of the sensitivities achieved for these PCRs along with PBS, PCs, and RCCs spiked with bacteria and for DNA template, is shown in Table 4.1. As little as 15 fg (1 genome) was detectable for *Y. enterocolitica*, whilst for *S. aureus* the smallest detectable amount was 143 fg of DNA (~80 genomes). Fewer than 30 bacteria in a sample could be detected from our spiked extracts. Importantly, although some difference in sensitivity was observed based on the source of the template (whether it was from bacteria spiked into PBS or blood products) it did not rise above the set limit of 10^3 CFU/mL. Sequencing of randomly selected samples showed that the correct target had been amplified in each instance. Non-target species did not produce amplicons, nor did the sterile no template controls (NTCs). Having shown that detection at this level can be reliably achieved within our specificity and sensitivity requirements, we examined whether the same could be achieved at the genus level.

4.3.2.2 Genus-specific targets – *Staphylococcus tuf* and *Yersinia ymoA* genes

For genus-specific detection of *Staphylococcus* and *Yersinia* spp., the *tuf* and *ymoA* genes, respectively, were amplified using published primers and methods (85, 127). Again, 10 µL of template was used in each reaction. Typical results for these are shown in Figures 4.6a and 4.6b.

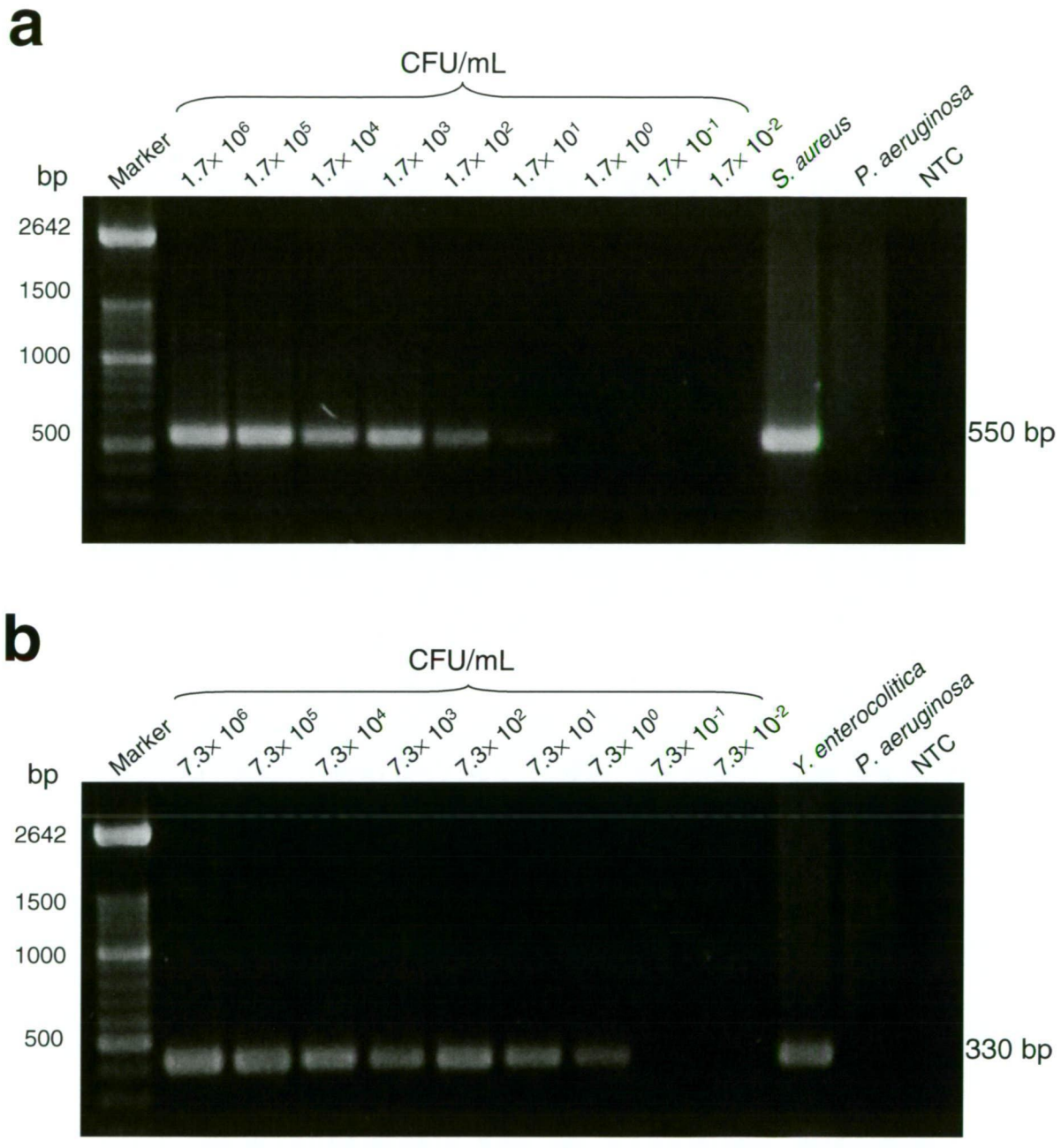


Figure 4.5: Representative species-specific standard PCR results

a) *mecA* - *S. aureus* in PBS. Pure *S. aureus* DNA was used as a positive control, whilst *P. aeruginosa* was a negative control. NTC; no template control.

b) *ail* - *Y. enterocolitica* in PBS. Pure *Y. enterocolitica* DNA was used as a positive control, whilst *P. aeruginosa* was a negative control.
Details of the primers and conditions can be found in Chapter 2, Section 2.8.2.3 and Table 2.3.

Table 4.1: Comparative sensitivities of species-specific PCRs

Data are the mean of two separate experiments. PCR samples were conducted in duplicate.

		CFU/mL		
	DNA	**PBS**	**PC**	**RCC**
Y. enterocolitica	15 fg	3.7×10^{0}	7.3×10^{1}	2.8×10^{1}
S. aureus	143 fg	1.7×10^{1}	7.6×10^{1}	1.5×10^{1}

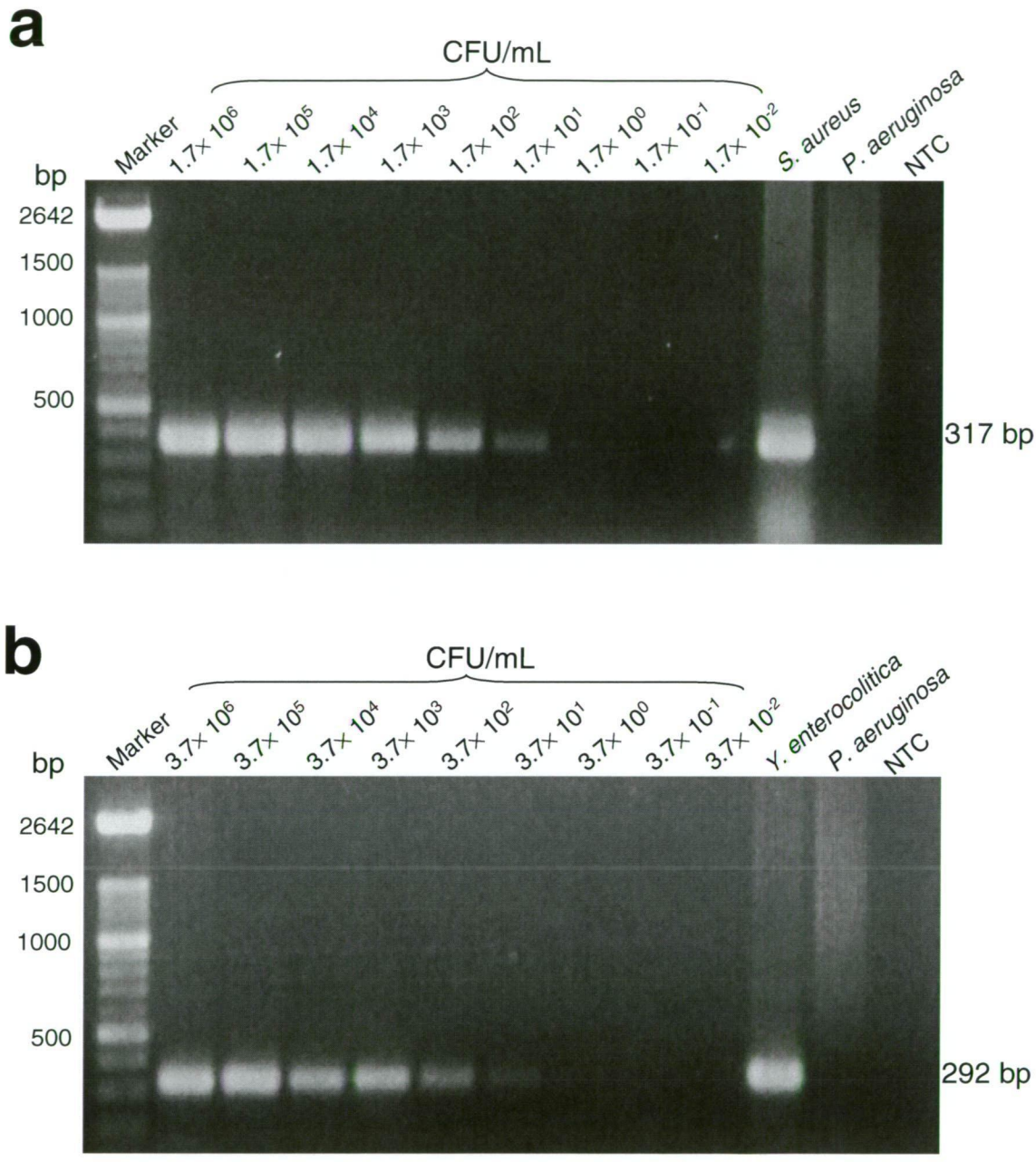


Figure 4.6: Representative *Yersinia* and *Staphylococcus* genus-specific standard PCR results

The source of DNA is shown above each lane.

a) *tuf* - *S. aureus* in PBS. Pure *S. aureus* DNA was used as a positive control, whilst *P. aeruginosa* was a negative control. NTC; no template control.

b) *ymoA* – *Y. enterocolitica* in PBS. Pure *Y. enterocolitica* DNA was used as a positive control, whilst *P. aeruginosa* was a negative control.
Details of the primers and conditions can be found in Chapter 2, Section 2.8.2.4 and Table 2.3.

A summary of the sensitivities achieved for these targets is shown in Table 4.2. Although the sensitivity appeared to be lower compared with the species-specific PCRs, the numbers of CFU/mL that were detectable were similar. The source of the template did not affect the sensitivity of the assay, and in each PCR, only members of the relevant genus produced amplicons of the expected size (Table 4.3). Random sequencing of these indicated that the intended target had been amplified in each instance. The NTCs were negative as expected.

As we had shown that PCR was successful using our template, without evidence of inhibition, or the template being overly sheared, we shifted our study to an investigation of 16S protocols. A semi-universal 16S PCR (Gram-positive or Gram-negative only) was evaluated initially; then a universal PCR to detect all bacterial species.

4.3.2.3 Semi-universal 16S PCR – Gram-positive only or Gram-negative only

As with the species- and genus-specific PCRs, the method and primers for the semi-universal detection of Gram-negative or Gram-positive species were obtained from published sources (88, 114). Both assays were targeted at a 313 bp region of the 16S gene. The Gram-positive PCR (Figure 4.7) successfully amplified all but one appropriate target (that being *Corynebacterium* sp., Table 4.4), without any false positive results from Gram-negative bacteria. However, many Gram-negative species failed to be identified by the Gram-negative PCR, and non-specific products of an unexpected size (indicated by white arrow in Figure 4.8) were present in some samples that contained Gram-positive template. Despite attempts to optimise the PCR by varying the primer, $MgCl_2$ concentrations the cycling conditions, it remained unreliable. Furthermore, as the Gram-specific PCRs utilised a small region of the 16S sequence, both produced occasional false positives (of the expected 300 bp size) in their NTCs (not shown).

Table 4.2: Sensitivities of *Yersinia* and *Staphylococcus* genus-wide PCRs

		CFU/mL		
	DNA	**PBS**	**PC**	**RCC**
Y. enterocolitica	15 pg	3.7×10^1	7.6×10^1	2.8×10^1
S. aureus	83 fg	1.7×10^1	7.6×10^1	1.5×10^1

Table 4.3: Specificities of *Yersinia* and *Staphylococcus* genus-wide PCRs

	Correct Genus	**Other Genus**
Yersinia	11/11	0/36
Staphylococcus	10/10	0/36

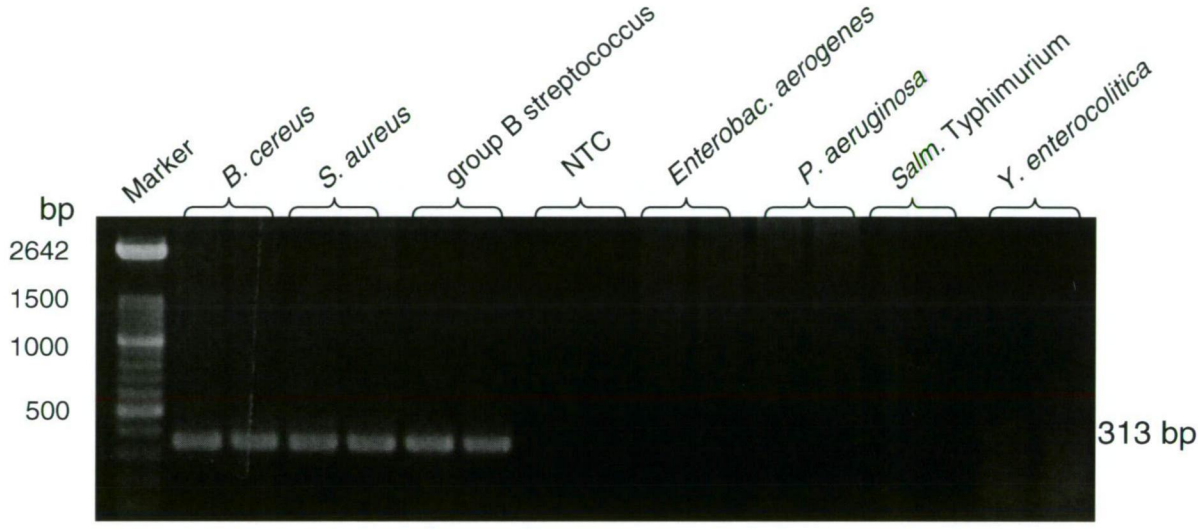


Figure 4.7: Representative results of generic PCR for Gram-positive bacteria

NTC; no template control.

Details of the primers and conditions can be found in Chapter 2, Section 2.8.2.5 and Table 2.3.

Table 4.4: Specificity of Gram-positive targeted PCRs

	Correct	Incorrect
Gram-positive spp.	10/11*	0/26

*Corynebacterium sp. was negative by this PCR.

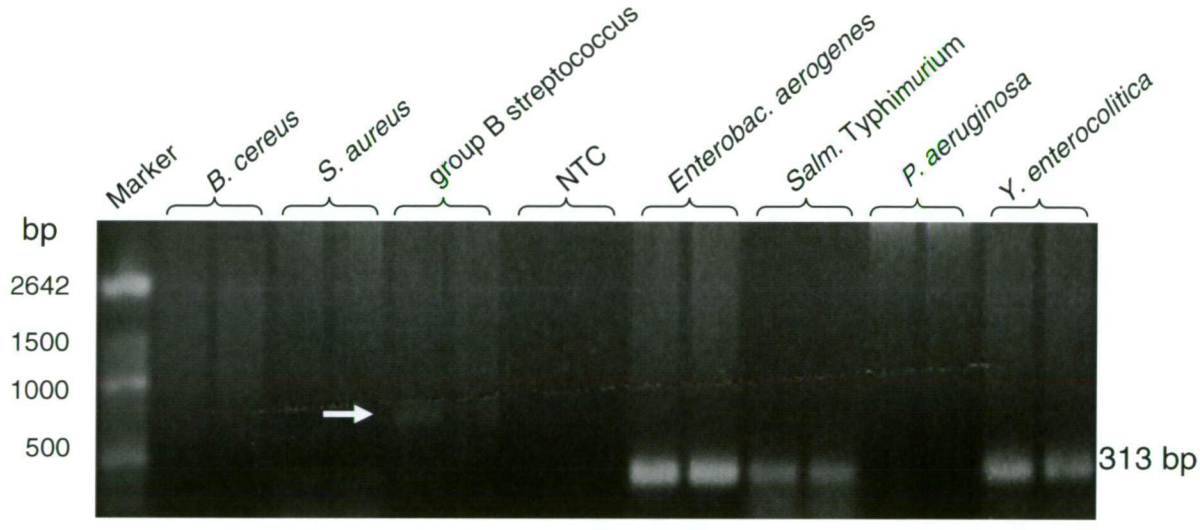


Figure 4.8: Representative results of generic PCR for Gram-negative bacteria

Unidentified non-specific amplicon indicated by white arrow.
The source of DNA is shown above each lane. NTC; no template control.
Details of the primers and conditions can be found in Chapter 2, Section 2.8.2.5 and Table 2.3.

Treatment of the PCR mastermixes with ultraviolet (UV) light prior to the addition of template (180) did not resolve this issue, nor did the strict control and replacement of reagents. Other published primer pairs and conditions were investigated (43) for detection of Gram-negative bacteria, but all produced similar or even less satisfactory results (not shown). For these reasons, we chose not to continue to our Gram-specific PCR experiments (in standard or real-time formats), but pursued a universal 16S PCR, instead.

4.3.2.4 Universal 16S targets – conserved 16S DNA regions possessed by all bacteria

Initially, our universal 16S PCR utilised published primers and targeted a 333 bp region of the gene (114). The PCR successfully amplified all 47 species and strains tested using 10 µL of template, showing it was capable of the universal targeting we had envisaged (Figure 4.9a). However, despite strict reagent handling measures and UV treatment of plasticware and the mastermix, false-positive reactions were a frequent occurrence (Figure 4.9b), and were observed in our NTCs and sterile PC and RCC extracts. We hypothesised that contaminating DNA was influencing these PCRs and producing false positive results, and that contamination of thus type could cause similar problems if this PCR was adopted by the transfusion industry.

Accordingly, we decided to target a larger fragment of the 16S gene (over 1000 bp), as many sources had reported that PCRs with amplicons of this size were significantly less vulnerable to producing false-positive results. The method and primers of Lane (1991) targeted a 1361 bp section of the 16S gene, and like our smaller fragment universal PCR, successfully amplified all 47 species and strains tested (Figure 4.10). Unlike the smaller universal PCR, however, no false-positive results were recorded in any assay.

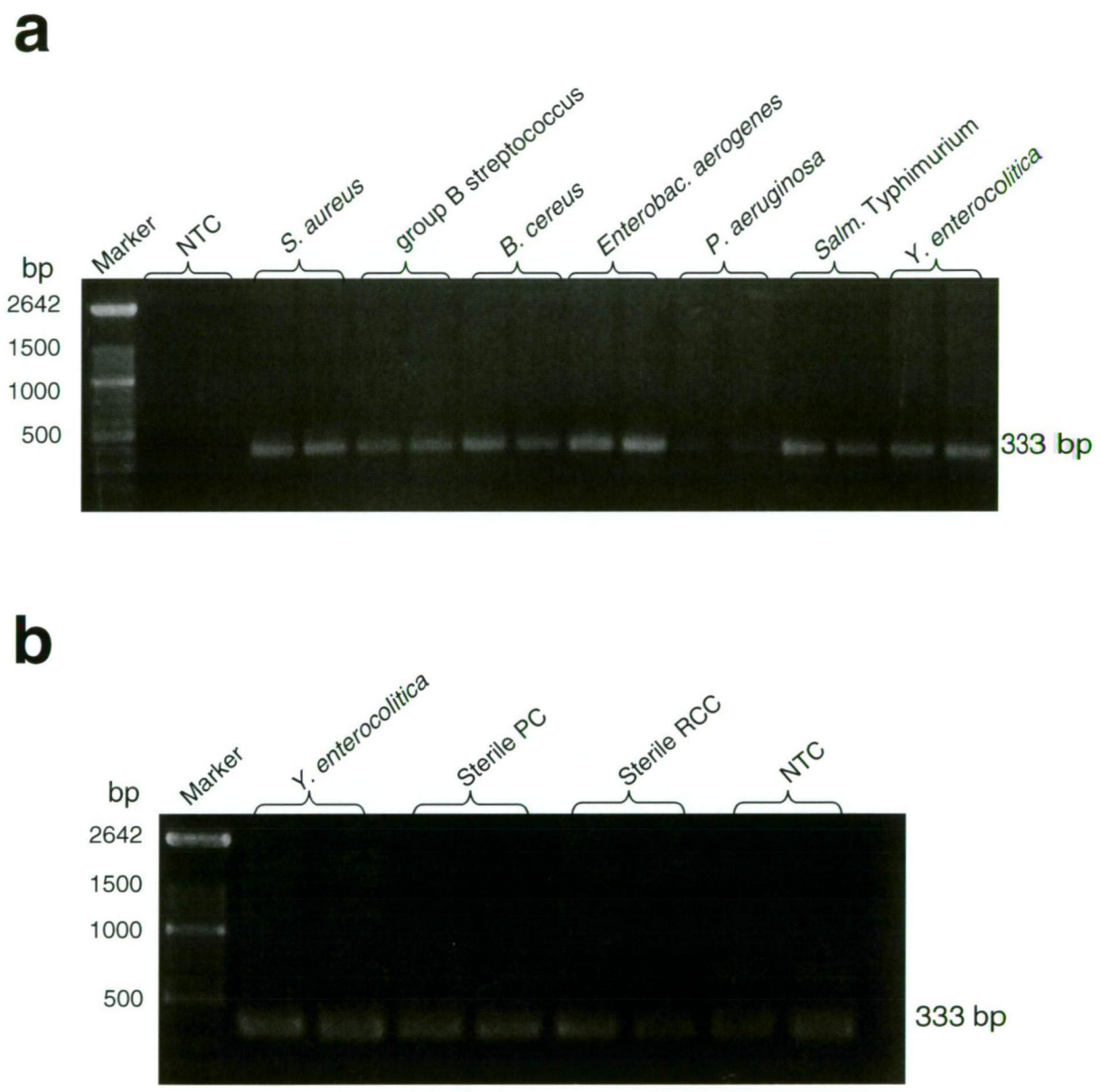


Figure 4.9: Representative results of universal 16S 333 bp PCR for bacteria

The source of DNA is shown above each lane.

a) correctly amplified products. NTC; no template control.

b) showing false-positive results from sterile PC, RCC, and NTC.

Details of the primers and conditions can be found in Chapter 2, Section 2.8.2.6 and Table 2.3.

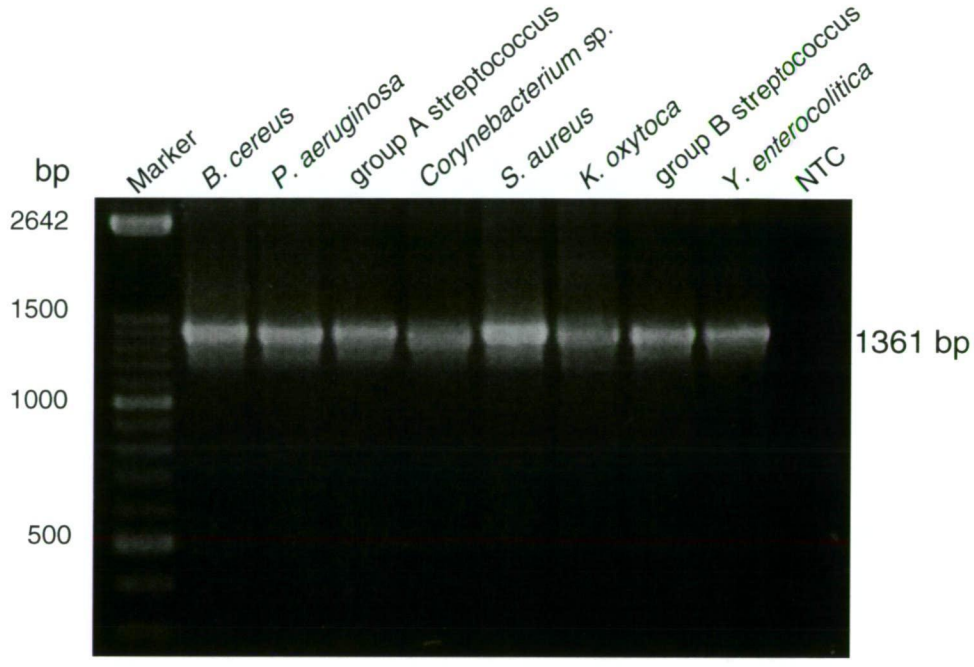


Figure 4.10: Representative results of 16S universal 1361 bp PCR

The source of DNA is shown above each lane.
NTC; no template control.
Details of the primers and conditions can be found in Chapter 2, Section 2.8.2.7 and Table 2.3.

Moreover, the sensitivity of each PCR was comparable to the species- and genus-specific PCRs, and was not influenced by the type of sample (Table 4.5). Random sequencing showed that the 16S gene had been amplified in each instance, giving the signature sequence of the expected bacterium (Table 4.6).

On the basis of these results this approach was adopted as the mainstay of the final screening method (a complete account of which is detailed in Chapter 5). However, investigations into the possibility of using real-time PCR were continued, because of its advantages in terms of speed and potential for automation.

4.3.3 Real-time PCR amplification

Like standard PCR, real-time PCR involves the use of primers and *Taq* polymerase to manufacture many copies of a target gene. Yet, unlike standard PCR, real-time PCR can be conducted in less than an hour due to the small (typically less than 200 bp) size of the target. Post-reaction electrophoresis is also unnecessary, because the reporting is conducted in 'real time' during the PCR, by fluorescent dyes such as SYBR green or fluorescently-labelled oligoprobes. As several PCRs were to developed, SYBR green was selected as the reporting tool, as this can be used for all reactions, and is not dependent on sequence-specificity as with the labelled oligoprobes. SYBR green operates by interchelating any double stranded DNA, so that as the amount of amplicon increases so does the fluorescent signal. Samples that do not produce amplicons do not fluoresce. When SYBR green is used as the reporter, presumptive identification of the product can be made post-cycling by a melt profile. The temperature of each sample is raised by 1°C increments, and as the amplicon melts, the SYBR green fluorescent signal is extinguished. As each sequence produces a signature melt profile, these allow for the presumptive identification of an amplicon (in the same way that the migration of an amplicon on gel electrophoresis post-standard PCR allows for presumptive identification of that product). Again, sequencing can be used to confirm the identity of the product.

Table 4.5: Sensitivity of universal 1361 bp 16S PCR for bacterial targets

		CFU/mL		
	DNA	**PBS**	**PC**	**RCC**
Y. enterocolitica	15 fg	3.7×10^{2}	7.6×10^{1}	2.8×10^{2}
S. aureus	1.4 pg	1.7×10^{2}	7.6×10^{2}	1.5×10^{2}

Table 4.6: Specificity of universal 1361 bp 16S PCR for bacterial targets

	Correct sequence	**Incorrect sequence**
Bacteria tested	47/47	0/47

NB: Sterile blood product extracts and NTCs were negative as expected.

4.3.3.1 Universal 16S targets – conserved 16S DNA regions possessed by all bacteria

The method of Nadkarni (143) was used to detect a 466 bp region of the 16S gene (Section 2.8.3.3). In their procedure, a 16S Taqman probe was used to confirm the amplicon was from a bacterial source. As the eventual goal was to individually identify the bacterial species in the PCR, we chose SYBR green as our reporter (with a view to develop the specific probes at a later stage, after showing the 16S real-time PCR was viable). Nadkarni used a commercially available mastermix, but a lab-made mix was initially used for these preliminary investigations. To ensure that an amplicon was produced, we first conducted trials using standard PCR and gel electrophoresis. Parallel assays were run, one of which contained SYBR green, and the other which did not. This was necessary to optimise the concentration of SYBR green needed for each reaction, since it is capable of binding the double-stranded template and inhibiting the reaction. At the manufacturer's recommended SYBR green concentration, no inhibition of the PCR process was noted. However, despite UV treatment of the mastermix, false-positive results were consistently detected from our NTCs (Figure 4.11a). This could not be resolved, despite implementing extended (1 min) UV treatment of our mixes and ensuring fastidious handling and preparation of the samples.

As an alternative, a commercially available mastermix was obtained from Invitrogen and the experiment was repeated with greater success. Three microlitres of template was used in each reaction, as greater amounts reduced the overall amount of amplicon produced, and also delayed the take-off point for the reaction by a number of cycles (not shown). Positive reactions were noted only when bacterial template was added to the mastermix and these were obtained for both trial species, *S. aureus* and *Y. enterocolitica* (Figure 4.11b). Figure 4.12 shows an example of the raw fluorescence, quantification, and melt profiles for a wide range of bacteria, using this PCR. All samples containing bacterial template were considered positive between 15 and 20 cycles of the program. All 12 species of bacteria tested were amplified by this method, with each producing slightly different melt profiles, due to sequence differences within each of their signature 16S gene sequences.

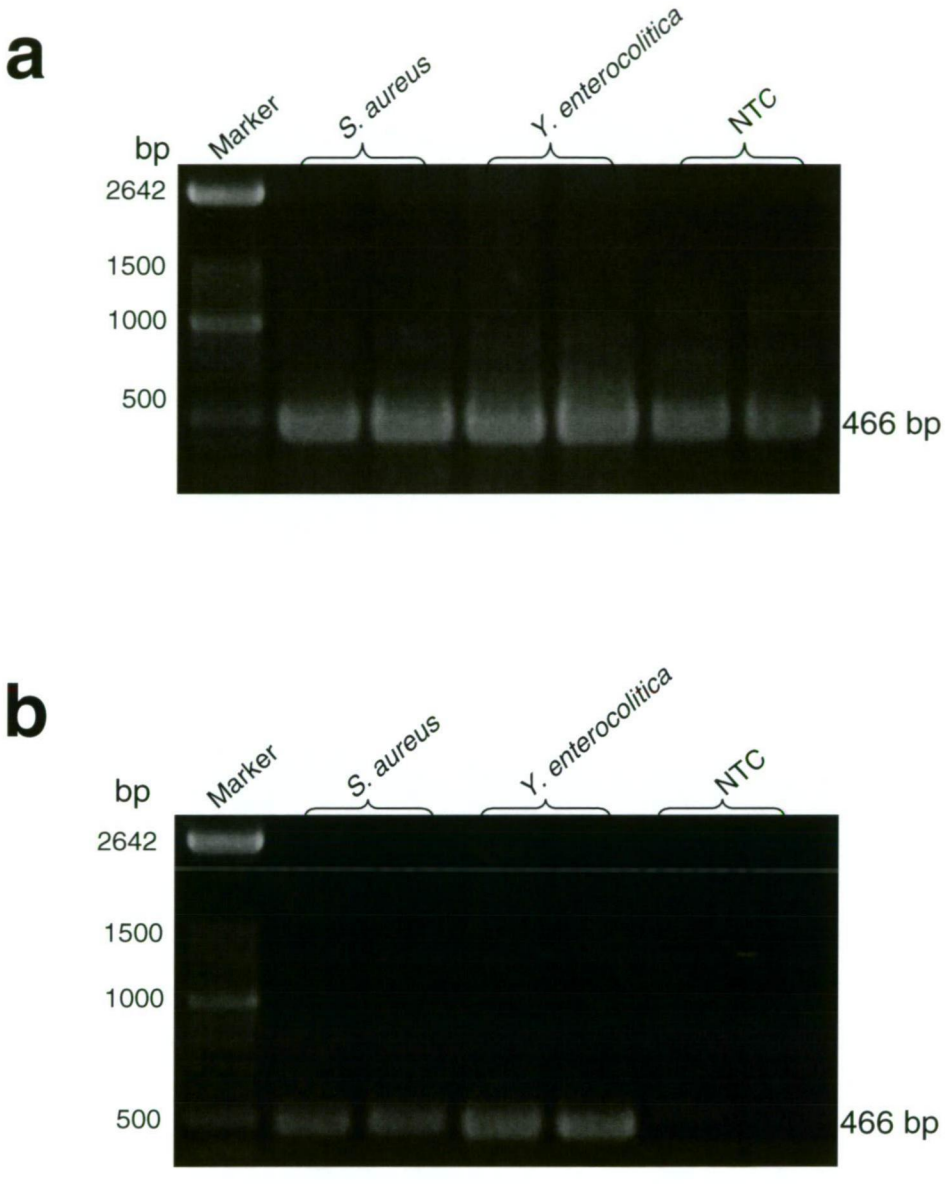


Figure 4.11: 16S Universal real-time PCR trial on standard equipment

The source of DNA is shown above each lane. NTC; no template control.

a) Home-made mastermix.

b) Invitrogen mastermix.

Details of the primers and conditions can be found in

Chapter 2, Section 2.8.3 and Table 2.3.

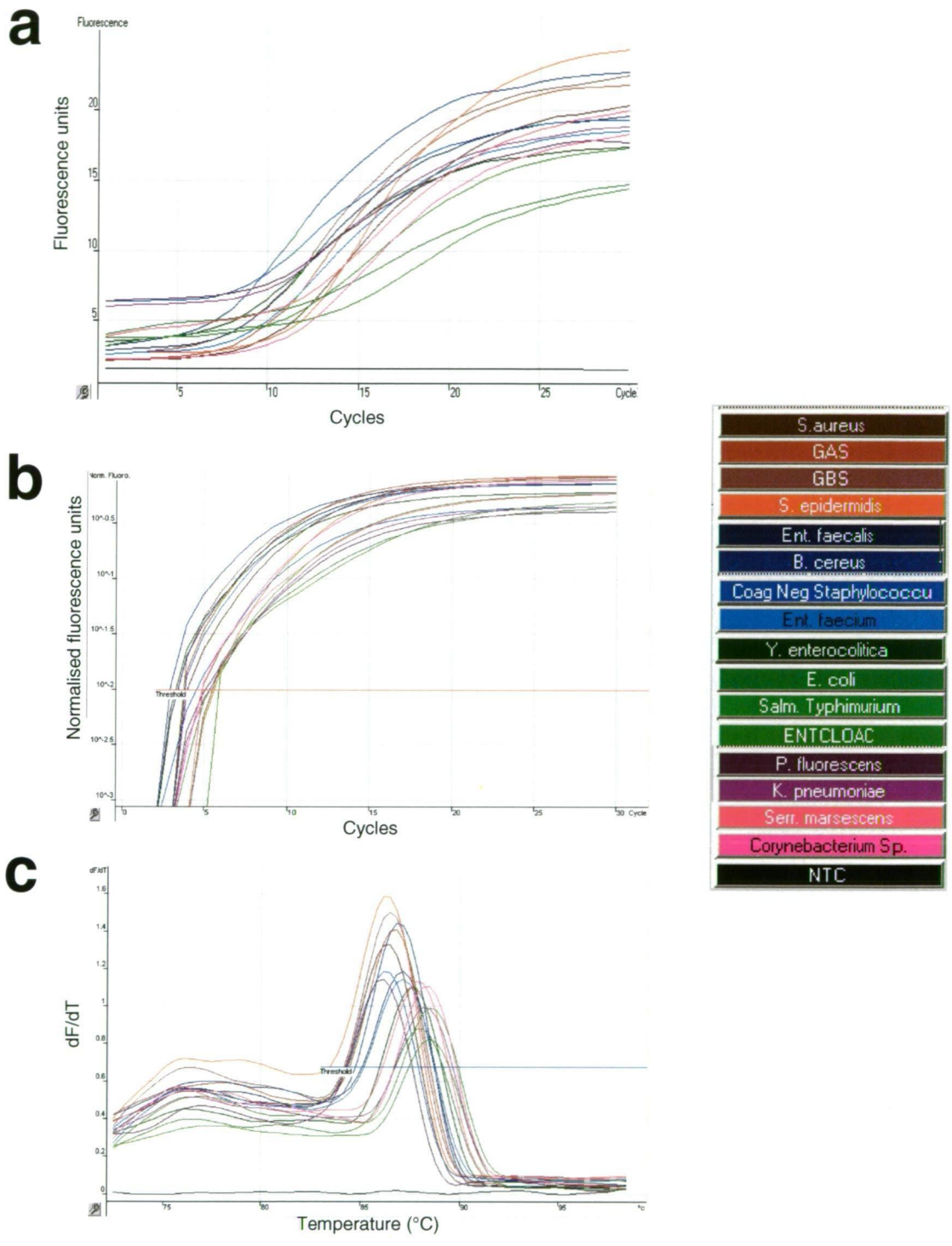


Figure 4.12: Representative real-time universal 16S PCR of a range of species

a) raw fluorescence of samples.

b) quantification (when samples become positive).

c) melt profile. dF/dT; differential (when amplicon dissociates). NTC; no template control.

However, this success could not be repeated with spiked PCs and RCCs which frequently gave a signal in the NTCs (not shown).

These experiments have shown that detection of bacterial DNA using real-time PCR and conserved 16S universal sequences in blood products may not be reliable, and that the method requires significant further optimisation. The success of 1.4 kb 16S PCR using standard equipment encouraged us towards investigating this technique thoroughly in order to achieve the aims of the project.

4.4 Confirmation and identification of bacteria post PCR

Once the ability to detect bacterial contaminants using standard 16S universal PCR in a sensitive, specific and timely manner was in place, it was important to confirm that the products detected were in fact 16S amplicons, and to determine if they could be used to identify the species of the bacterial contaminant.

Confirmation of PCR products may be achieved by several techniques. Sequencing of the 16S gene is commonly used when identifying unculturable bacteria. However, sequencing is expensive and impractical for the number of samples that would need to be processed for blood product screening. Instead we decided to confirm and identify these PCR products using oligonucleotide probes targeted to the variable regions of the 16S gene, and compare the efficacy of PCR-ELISA and Southern dot blotting for this purpose.

4.4.1 PCR-ELISA

Provided as a kit, PCR-ELISA uses biotinylated oligonucleotide probes to hybridise to DIG-labelled PCR products, with the hybrids detected by using an anti-DIG antibody and peroxidase colour reaction. The assays are carried out in streptavidin-coated 96-well ELISA trays, with the oligoprobes capturing the samples in the wells. This allows testing of many probes simultaneously, in a macroarray arrangement. PCR-ELISA has been used previously to detect viral, bacterial, and eukaryotic DNA, from a range of sample types such as food, serum, and tissue biopsies (184, 198, 228). The oligoprobes selected were of similar melting temperatures, with the aim that all might be used simultaneously. An example of the DIG-labelling efficiency of the samples is shown in Figure 4.13.

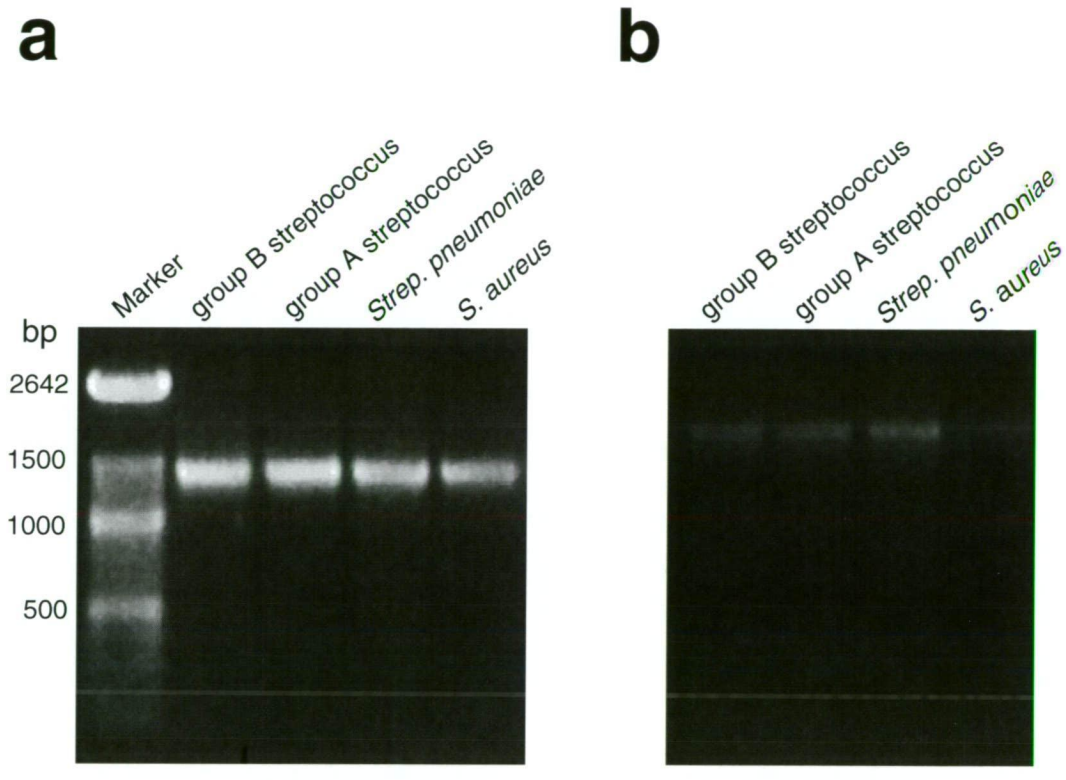


Figure 4.13: DIG-labelling by PCR of 16S product for detection by PCR-ELISA

The source of DNA is shown above each lane.

a) Unlabelled PCR product.

b) DIG-labelled PCR product.

A graph of typical results (averaged from duplicate samples) using the PCR-ELISA is shown in Figure 4.14a.

It was apparent that despite strict adherence to the assay protocol, there was considerable non-specific binding of the anti-DIG antibody to samples that did not contain DIG-labelled PCR product. After investigating the available literature, a blocking step using a PBS/skim milk buffer was implemented prior to applying the anti-DIG antibody to the samples. This approach significantly reduced the background (Figure 4.14b), but the strength of positive signals was not of the same order as those obtained when using the DIG-labelled PCR and the probe supplied with the kit. Despite many attempts to optimise this method by varying the annealing temperature of the assay and using a range of probes, detection could not be improved. Furthermore, repeat assays under identical conditions produced variable results (Figure 4.15), and it became apparent that this method was not consistent or reproducible. Therefore, Southern dot blotting was investigated as an alternative detection method.

4.4.2 Southern dot blot

Southern dot blotting is a technique by which sequences can be identified through hybridisation with a probe of a known sequence. It can be performed in two orientations: with the DIG-labelled probes of known sequence in solution, and the unidentified sample bound to a membrane (standard orientation), or whereby the DIG-labelled samples are in solution, with the probes of known sequence bound to the membrane. Each technique has advantages and disadvantages compared with the other. The standard orientation allows for many samples to be examined with a single probe, whilst reverse-probing allows a single sample to be tested against many probes simultaneously (similar to the PCR-ELISA approach). The aim was to compare and contrast both forms of Southern dot blot and to establish which was more suitable.

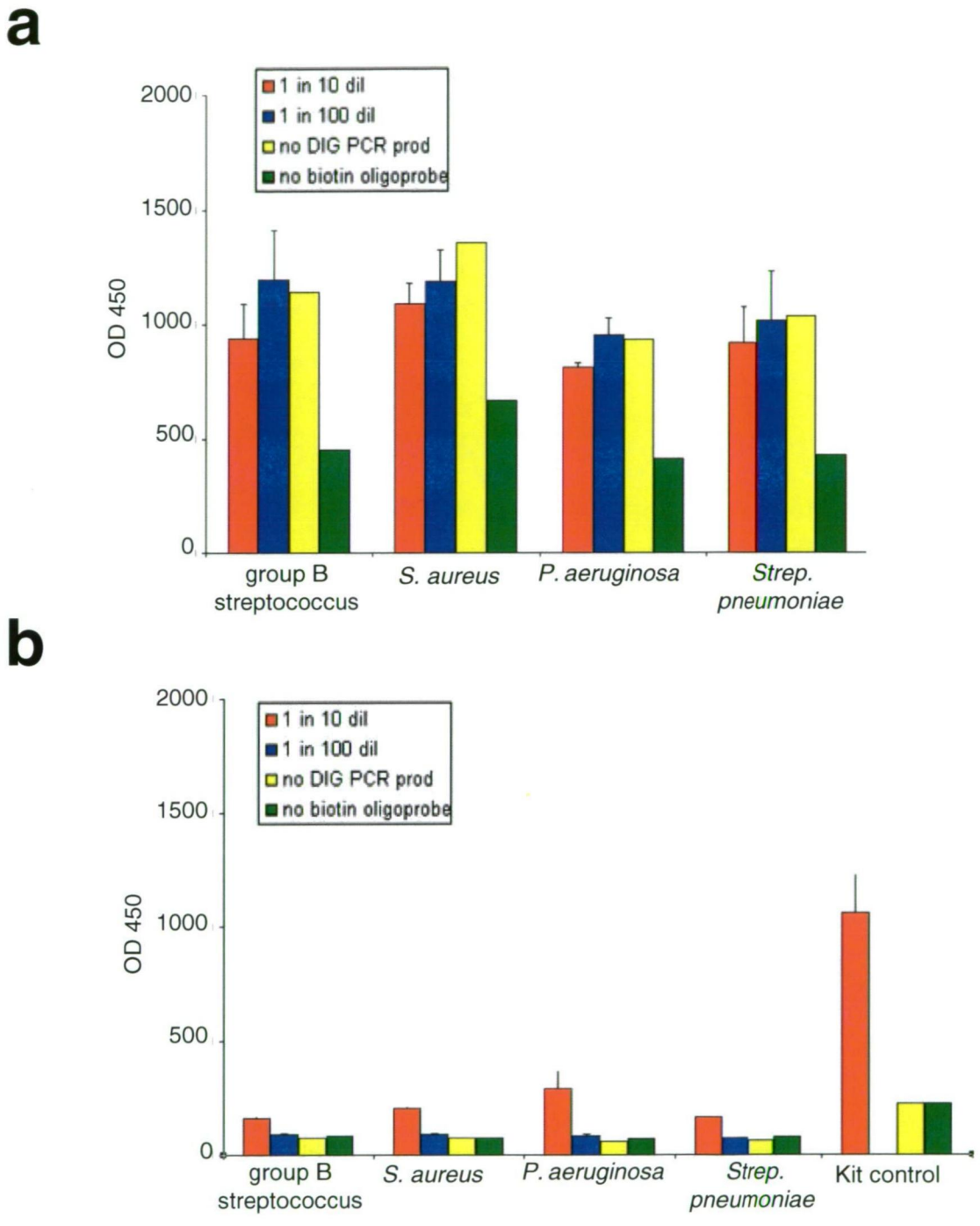


Figure 4.14: Example data obtained by PCR-ELISA detection of DIG-labelled 16S PCR products

a) standard kit protocol.

b) modified blocking protocol
dil; dilution of DIG-labelled 16S PCR product. NTC; no template control. All results are the average of duplicate wells.

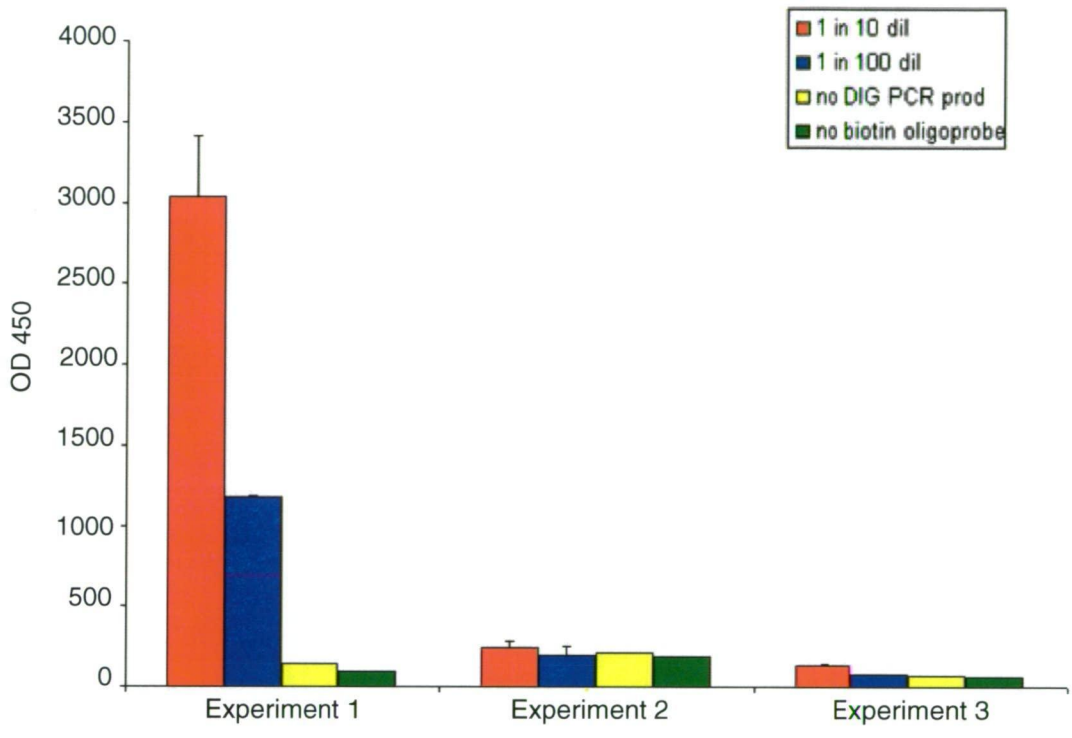


Figure 4.15: Detection of *B.cereus* by PCR-ELISA – variability between experiments

Note: same conditions, DIG-labelled PCR product and, amount of oligoprobe used in all experiments. dil; dilution of DIG-labelled 16S PCR product. NTC; no template control. All results are the average of duplicate wells.

4.4.2.1 Reverse dot blot

We have already shown that conserved bacterial 16S sequences from a diverse range of species can be amplified from pure cultures and spiked blood products with a high degree of specificity and sensitivity. Oligonucleotides were selected as probes, as these are able to differentially target the unique and variable 16S sequences more specifically than larger probes. Instead of designing these probes to be of similar annealing temperatures, we selected or designed them to be 18-mer lengths, and to use buffers containing the quaternary ammonium salt tetramethylammoniumchloride (TMAC) to stabilise the A-T, and G-C bonds to be of equal melting temperature (Tm). These buffers allow hybridisation of the target and probe to occur strictly by sequence matching (102, 130, 222). The probes were spotted onto a positively charged nylon membrane at a range of concentrations, as were unlabelled positive and negative control PCR products. Hybridisation of the DIG-labelled PCR product to the membrane-bound probes was optimised over a range of temperatures (40 to 60°C), but none produced positive signals except where the DIG-labelled PCR product had bound to its unlabelled equivalent (an example of which is shown in Figure 4.16a). Furthermore, hybridisation temperatures below 50°C yielded non-specific interactions (and hence false-positive results) between sterile RCC extracts and the anti-DIG antibody (Figure 4.16b). We examined the need for purification of these PCR products generated from RCC extracts with the QIAquick PCR purification kit, but found this step was unnecessary and that hybridisations and washes conducted at 50°C were sufficient to prevent non-specific interactions.

To determine if the oligoprobes were bound to the membrane throughout probing and detection at this temperature, some probes were DIG-tailed, applied to the membrane and detected directly. Figure 4.16c shows an example of the probes stably bound and detected at this temperature. Some investigations have utilised poly dT tailing with short oligoprobes (such as those used here) to enhance their binding efficiency (107, 178, 229). This technique was successfully applied to the *S. aureus*-specific probe (Saur2) for 1 h (not shown), but this made no difference to our ability to detect the DIG-labelled PCR product (Figure 4.17).

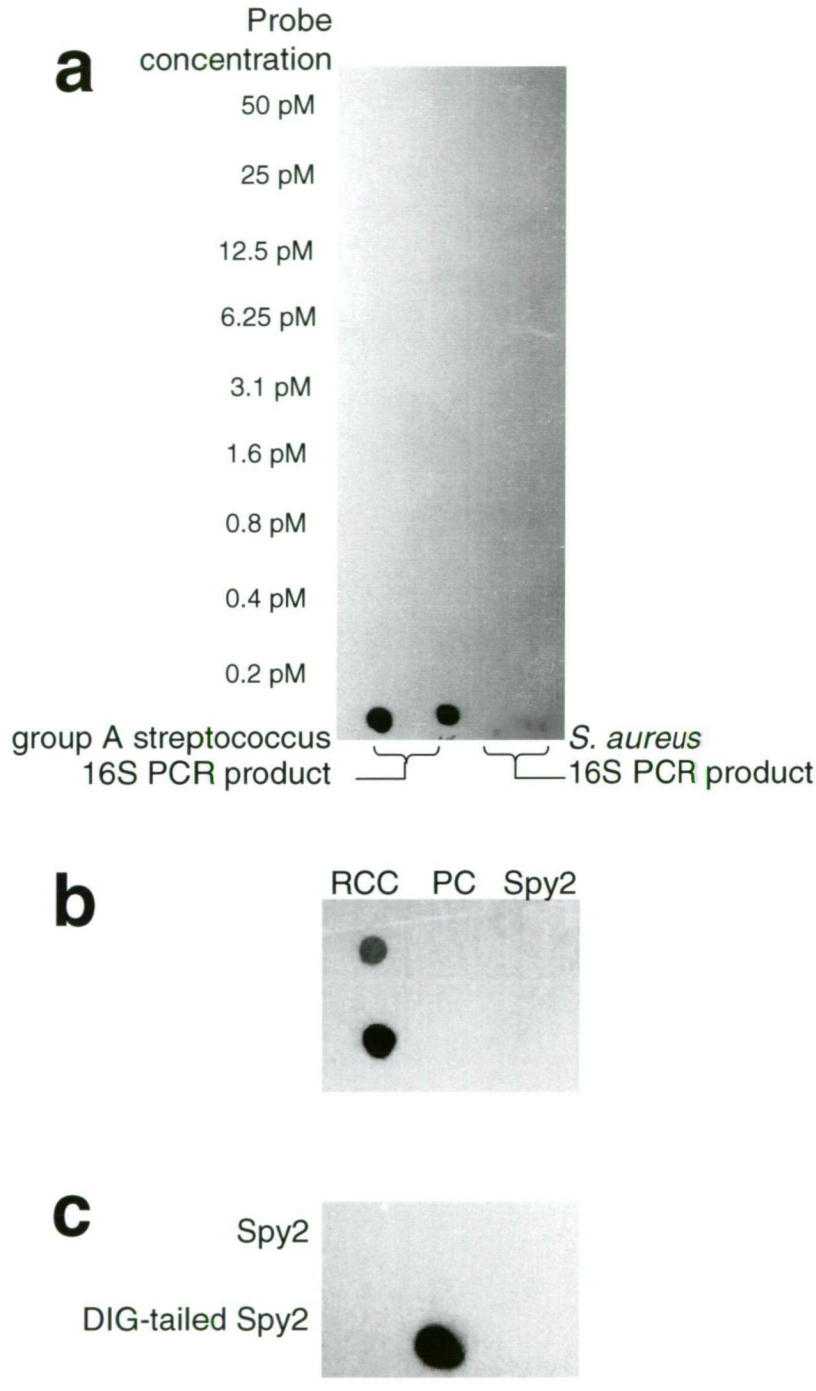


Figure 4.16: Representative reverse dot blots of 1361 bp PCR product

a) Hybridisation at 55°C of group A streptococcus specific probe (Spy2).

b) non-specific binding of sterile RCC extract to anti-DIG antibody at 55°.

c) oligoprobes remain bound to membrane throughout probing, washing and detecting.

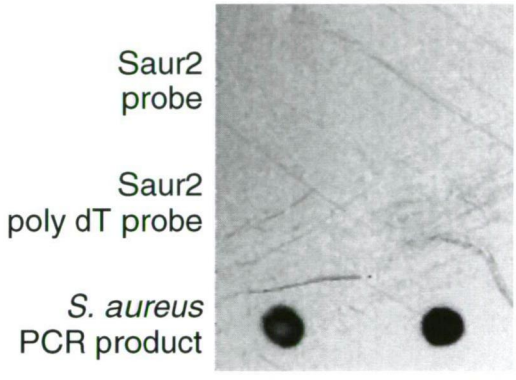


Figure 4.17: Effect of poly dT tailing of on the ability to detect *S. aureus* using a *S. aureus*-specific probe

Reverse dot blot of membrane bound poly dT tailed and untailed Saur2 probe and 16S PCR product to DIG-labelled *S. aureus* 16S product

All samples spotted in duplicate.

A range of tail lengths were then explored to determine if another would be suitable. In addition, DIG-labelled PCR samples were digested with *Rsa*I, in the event that the 1400 bp product was too large for an 18-mer probe to bind, or if the homologous binding site was masked by the tertiary structure of the 16S molecule. Although complete cleaving of the PCR product was achieved (not shown), this still did not allow successful hybridisation, nor did the range of dT tail lengths have any effect (Figure 4.18).

Results thus far using reverse dot blot did not suggest this technique was going to be successful in this instance, so the standard orientation was investigated instead.

4.4.2.2 Standard dot blot

As the reverse dot blotting experiments showed a tendency for non-specific interactions to occur between sterile RCC extracts and the anti-DIG-antibody at hybridisation temperatures below 50°C, probes were selected or designed to hybridise at this temperature. For standard dot blotting, the oligoprobes were DIG-tailed and diluted to the recommended concentration in DIG Easy-Hyb buffer. A range of 16S universal PCR products from different species was spotted onto the membrane, and allowed to hybridise with the DIG-labelled oligoprobes. Detection of the probe-PCR hybrids was successful at the first attempt, and no false-positives were detected in sterile blood product extracts (Fig 4.19). The sensitivity of detection was reliant upon the initial generation of the PCR product. Therefore, the sensitivities of the standard dot blots were the same as those for the PCR itself (Table 4.5). Despite careful design of each probe, some resulted in non-specific hybridisation (Figure 4.20a), as could be expected from targeting a 16S sequence which contains many shared sequences between species. However, careful re-designing of the probes allowed the desired result to be obtained (Figure 4.20b).

The success achieved by the standard dot-blotting procedure provided us with a mechanism for detecting and identifying bacterial contaminants in blood products. This technique was, therefore, selected for further investigation, as described in Chapter 5.

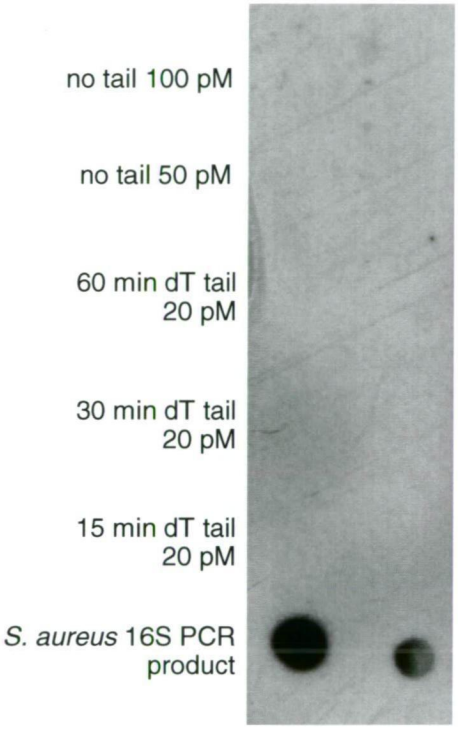


Figure 4.18: Reverse dot blot of *Rsa*I digested, DIG-labelled *S.aureus* 16S PCR product, with the oligoprobe Saur2 poly dT tailed at a range of lengths

All samples spotted in duplicate.

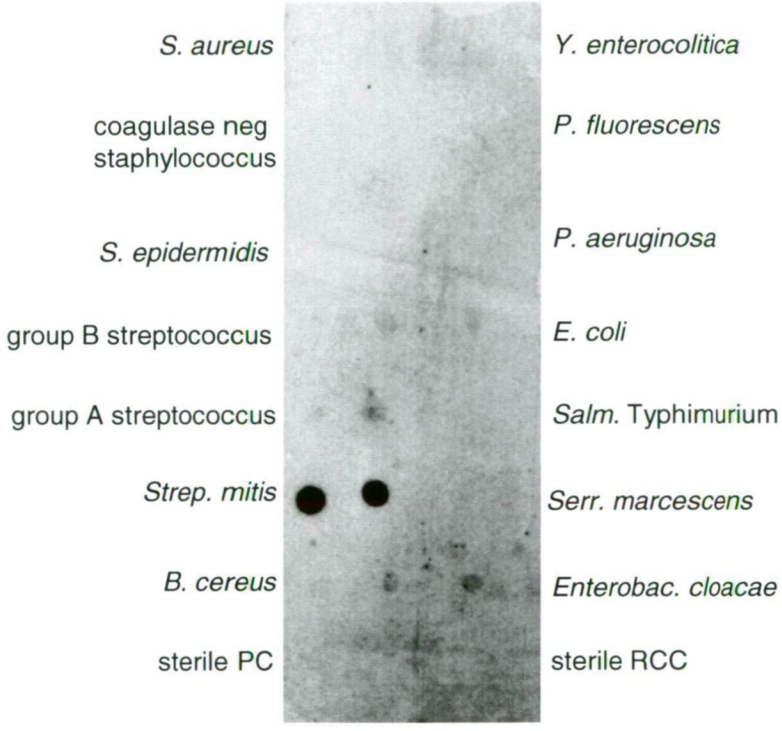


Figure 4.19: Representative standard dot blot of *Strep. mitis* 16S PCR product with the probe STREP16SR

All samples spotted in duplicate.

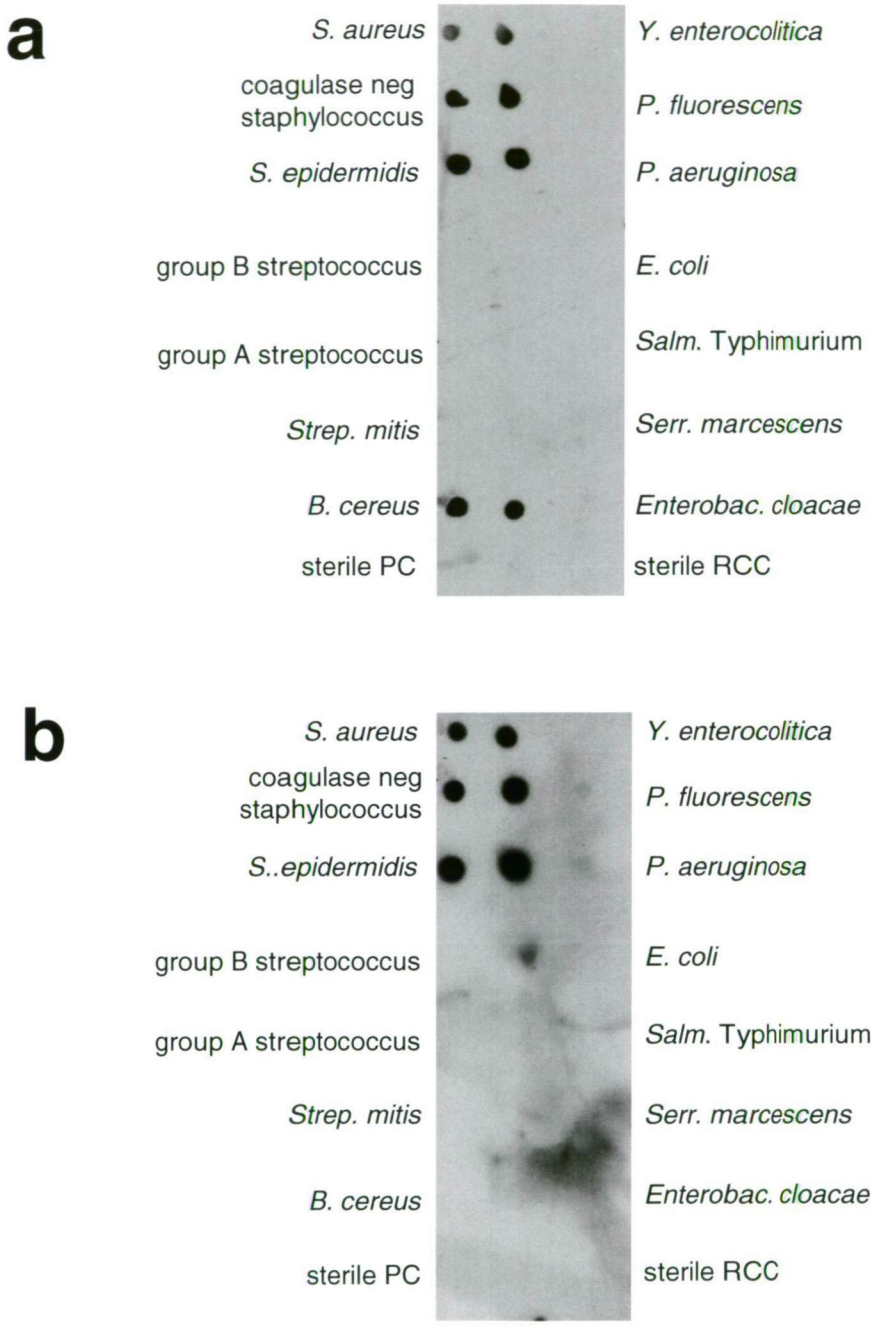


Figure 4.20: Representative standard dot blots of the *Staphylococcus* genus probe Staph3

a) Staph1 probe showing non-specific detection of *B. cereus*

b) Staph3 probe (redesigned from Staph1) showing detection of the staphylococci only

All samples spotted in duplicate.

4.5 Discussion

With the continuing threat of bacterial contamination of the blood supply, and the absence of feasible measures to detect and prevent the administration of many of these contaminated products, the aim of this study was to develop means by which all bacterial contaminants could be detected and identified in blood products. A number of studies have shown that PCR of 16S sequences can be used to detect bacteria in clinical and environmental samples (143, 162), and this study has shown that it is also possible to detect a broad variety of contaminants in blood products, in the same way. Furthermore, contamination can be detected before the numbers of bacteria reach what are considered to be clinically relevant numbers, and before non-invasive methods such as visual inspection for spoilage of the product can detect them. For efficient and rapid extraction of our samples we developed a novel method, as commercially available kits could provide the required speed or use the required sample volume. Confirmation of true positives and identification of the contaminant(s) responsible was achieved by Southern dot blot analysis with an array of specialised oligoprobes. This complete technique may prove useful in a range of clinical and diagnostic settings, apart from the screening of blood products.

The foundation for the ability to detect bacterial contamination in blood products containing less than 10^3 CFU/mL rested with the ability to extract DNA from each of the diverse array of bacteria typically found in PCs and RCCs quickly, efficiently, and cleanly, without introducing extraneous contaminants. Extraction kits are at the forefront of molecular clinical diagnoses, due to their simplicity and consistent results and were, therefore, our first choice for examination. However, neither of the kits trialled could process our target volume of 1000 µL, nor were they able to remove enough of the considerable particulate matter from RCC samples. A prelysis, step such as that used in our method, may provide an opportunity for processing of larger samples and a greater ability to remove cellular debris prior to the extraction process itself. However, the reliance of the Qiagen kit upon the enzymatic lysis of bacteria would still make this method too slow for this application. A bead-milling process similar to that used by BIO101 is available from Qiagen, and would be worthy of investigation in the future. The novel extraction method developed in this Chapter combining bead-milling and spin column cleanup,

provided a sample suitable for analysis in under 2 hours. Although some template shearing occurred due to the nature of the milling process, this had no effect on the ability to amplify targets of up to 1.5 kb, with a sensitivity within our cut-off of 10^3 CFU/mL. Furthermore, the DNA purified by this method could be used in several downstream applications such as PCR (both standard and real-time) and Southern dot blot. Further investigations are currently underway to apply this method to a greater range of sample types (e.g. tissue) and bacterial species (e.g. *Mycobacterium* spp.), and to expand the range of applications to which the purified template can be applied, such as DNA cloning. Other advantages of this method are that it requires no hazardous phenol/chloroform, and that many samples can be processed simultaneously. The rate-limiting step of the procedure is the Proteinase K treatment which requires 1 h incubation. Further optimisation of this method should include investigating the possibility of shorter incubation times, or alternative methods to reduce the protein content of samples.

Diagnostic laboratories have increasingly adopted PCR to detect the cause of disease in a variety of circumstances (60, 126, 176). PCR has been particularly helpful in identifying pathogens and environmental commensals, where traditional methods such as culture have failed. Numerous recent publications have explored the use of conserved bacterial 16S sequences to detect bacteria from a variety of sources, although none has applied this technology to bacteria in blood products. Template produced by the novel method presented here could be used in PCR for targets of at least 1.5 kb, and allowed specific and sensitive detection of less than 100 bacteria from pure cultures and each of the blood product types tested. Furthermore, the sensitivity was approximately the same for both standard and real-time PCR, despite the differences in the amount of template used, and the way in which a positive result was identified.

As previously reported, the success of universal 16S PCR is determined by the size of the target. Numerous publications have highlighted the issue of contaminating environmental DNA, and its propensity to give false-positive results where small PCR targets are used (163). The source of such contamination can be from any of the reagents used in the extraction or the PCR process, or it can surface from the samples themselves, which may be sterile but contain bacterial DNA (129).

Fastidious handling of reagents and samples (as in this study) is not always fail-safe, nor is the UV treatment (to crosslink any endogenous contaminating nucleic acid) of plasticware and reagents prior to the addition of template. Some investigators have advocated treating the PCR mastermixes with restriction enzymes or DNases (42, 54, 185). However, as these enzymes are from bacterial sources (and hence may contain bacterial DNA), they can exacerbate the problem of false-positive reactions (94). We found that the use of commercially available PCR mastermixes reduced (but did not eliminate) the incidence of false-positives during real-time PCR. These may also be useful in standard PCR. The future availability of 'DNA-free' plasticware and reagents should also have a positive effect on the ability to perform smaller fragment 16S PCR. In this study, however, the most successful approach to eliminating the problem of false-positives during universal PCR was to target a large (1.4 kb) region of the 16S gene. The basis for the success of this method is that most environmental DNA is much smaller than this target size and therefore cannot be amplified. Studies of bacteria spiked into blood products showed that this PCR was able to reliably and detect all of the diverse species of bacteria tested by this assay with a sensitivity below the target of 10^3 CFU/mL.

Following the successful universal amplification of bacterial DNA, the next task was to confirm the positive PCR reactions and identify the bacterium responsible. Several techniques were compared, the most successful of which was Southern dot blotting with labelled oligoprobes.

Although PCR-ELISA has previously been used to detect a range of infectious agents from clinical and food samples (184, 198, 228), in this instance the oligoprobes failed to hybridise with the target PCR product, despite many attempts to optimise the method. This may have been due to an incompatibility in probing a 1361 bp sequence, with a 20-mer probe. Generally, published probes used in PCR-ELISA are between 10 and 20% of the length of the sequence they are targeting, which would suggest that in order to detect our large PCR product, a probe of at least 140 bp would be required. The alternative approach (i.e. generating a smaller PCR product in order to use these existing probes), was not feasible, as PCRs which generate low molecular weight products tend to give false-positive results, such as occurred with the 300 bp universal PCR trialled in this study. Furthermore, DIG-

selected genera, the family Enteribacteriaceae, Gram positive or Gram-negative species, or all species, with a universal 16S probe (details of which are provided in Chapter 5).

In summary, sample extraction using a bead-beating, deproteination and spin column purification followed by large-fragment 16S universal PCR, and standard Southern dot blotting was the most successful method for detecting contamination in blood products. The preliminary success with this method laid the basis for further investigations with a large range of bacteria, further probe development, and determination of the ability of this method to be used with 'real' samples, taken from contaminated products.

CHAPTER FIVE

DETECTION OF BACTERIAL CONTAMINANTS IN BLOOD PRODUCTS USING UNIVERSAL 16S PCR AND SOUTHERN BLOT

5.1 Introduction

We have shown that it is feasible to use 16S universal PCR and Southern dot blot to identify contaminated products specifically and sensitively, whilst confirming the positive result and identifying the contaminant. Therefore, it was warranted that the repertoire of probes be expanded and tested on pure cultures, spiked PCs and RCCs. Further validation of the protocol with samples kept from the growth kinetic studies described in Chapter 3 was also desirable, to determine if this method performs differently with bacteria that have been allowed to proliferate in stored products over time.

5.2 Selection, extraction, PCR and Southern dot blot detection of samples

Initially, pure cultures and spiked blood products were used to evaluate the extraction, PCR and the Southern dot blotting processes.

5.2.1 Selection of frozen, growth-kinetic samples for testing

For the growth kinetic experiments (detailed in Chapter 3), the samples from the *Y. enterocolitica* PC and RCC experiments (starting spiking numbers of 10^1 and 10^2 CFU/mL) were selected for study. The replicates from each day's sampling were pooled (i.e., the five 0 d samples were pooled together, the five 1 d samples, and so on), as we knew the number of CFU/mL in these. Following thorough mixing, 1000 µL aliquots were taken from each day's pooled samples and extracted using the novel developed method (Section 4.2.3), before elution into 100 µL AE buffer.

5.2.2 Preparation of spiked PC and RCC samples for testing

A range of bacterial species were spiked at a concentration of approximately 10^9 CFU/mL into PC and RCC samples, and extracted by our novel rapid method. Spiking numbers were checked by plate count and were between 10^8 and 10^9 CFU/mL.

5.2.3 PCR of samples

Ten microlitre aliquots of template (extracted from frozen samples, spiked PCs and RCCs and pure cultures) were subjected to 50 cycles of the 1361 bp 16S universal PCR. Ten microlitre PCR samples were checked by gel electrophoresis for the presence of amplicons of the expected size. Half of each remaining RCC PCR sample was purified by the QIAquick PCR purification kit.

5.2.4 Selection of oligoprobes

Oligoprobes were selected according to their anticipated target. Some were from published sources, and others were designed in this study using the CLUSTALW and BLAST algorithms (Table 2.5).

5.2.5 Southern dot blot detection of bacteria using oligoprobes

The complete method is described in Chapter 2 (Section 2.11.3), but briefly, the oligoprobes were DIG-tailed and diluted in DIG Easy Hyb buffer, according to the manufacturer's recommendations. One microlitre aliquots of PCR product were spotted in duplicate onto nylon membrane, along with the appropriate negative (sterile PCs and RCCs) and positive (pure culture PCR product) controls. Hybridisation and stringency washes were conducted at 50°C, and detection was by CPD-Star chemiluminescence.

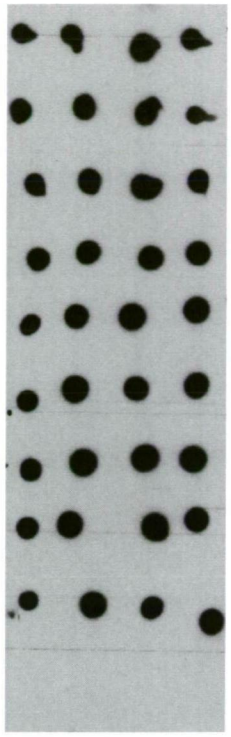
5.3 Southern dot blot of PCRs from pure cultures and spiked PCs and RCCs

Of more than 50 probes investigated, 14 were found to be useful.

5.3.1 SP16SR - 16S Universal probe

First published by Hendolin (90), this generic 16S primer was found to be a useful universal probe, as it detected all bacterial species that it was tested against, and it did not cross-react with human DNA (from sterile PC and RCC extracts). The specificity of this probe was the same with all sample types (PCR products generated from pure cultures and spiked PCs and RCCs). An example blot and a summary of results obtained for this probe are presented in Figure 5.1.

a



b

Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	+	+	+
Enterococ. faecalis	+	+	+
Enterococ. faecium	+	+	+
S. aureus	+	+	+
S. epidermidis	+	+	+
Coagulase negative staphylococcus	+	+	+
group A streptococcus	+	+	+
group B streptococcus	+	+	+
Strep. mitis	+	+	+
Strep. pneumoniae	+	NT	NT
Gram-negative			
Enterobac. aerogenes	+	NT	NT
Enterobac. cloacae	+	+	+
E. coli	+	+	+
E. coli (β-lactamase)	+	NT	NT
K. pneumoniae	+	NT	NT
P. aeruginosa	+	+	+
P. fluorescens	+	+	+
Salmonella Typhimurium	+	+	+
Serr. marcescens	+	NT	NT
Y. enterocolitica	+	+	+

Figure 5.1: SP16SR universal 16S probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

5.3.2 143+3 - Gram-positive probe

The Gram-positive probe used in this study was based on an oligonucleotide used for PCR of Gram-positive species by Klausegger (114). The original published probe (143 , see Table 2.3) was trialed for use, but the positive signals (although correctly targeted) were weak (not shown). Several probes based on this sequence were designed, and probe 143+3 was selected as it gave strong positive signals with the presence of Gram-positive target, but not with non-targeted (Gram-negative and sterile PC and RCC controls) samples. The specificity of this probe was the same for all sample types, and an example blot and a summary of results for 143+3 are presented in Figure 5.2.

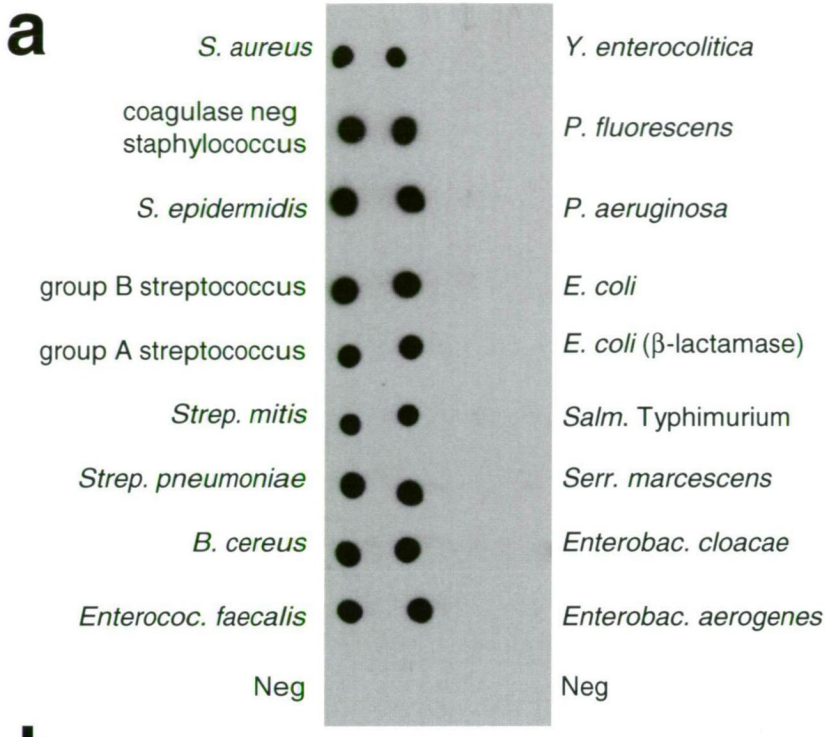
5.3.3 Yent2/N6R - Gram-negative probe

Several published Gram-negative probes were selected for study, the most successful of which was N6R (43), which reliably detected all Gram-negative species, except *Y. enterocolitica* (not shown). Several new probes were derived from this original sequence. However, these were also unable to detect *Y. enterocolitica*, or gave rise to significant cross-reactions with Gram-positive species (not shown). It was then decided to combine equal quantities of N6R and Yent2 (which is a *Y. enterocolitica*-specific probe, discussed in Section 5.3.12) and apply this as the Gram-negative probe. This approach proved highly successful and detected all targeted species, without any non-specific activity, and regardless of sample type. This also showed that it was possible for several probes to be combined, rather than use a single probe for each blot. An example blot and a summary of results obtained for this probe are presented in Figure 5.3.

5.3.4 Staph3 - *Staphylococcus* genus probe

The Staph3 probe is novel to this study, and was produced using CLUSTALW alignments and BLASTN searches. Four novel probes were developed for investigation, but not all were found to be specific or as sensitive as intended (See Figure 4.20a for an example).

a



b

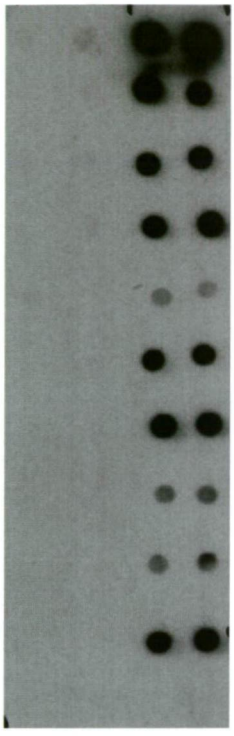
Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	+	+	+
Enterococ. faecalis	+	+	+
Enterococ. faecium	+	+	+
S. aureus	+	+	+
S. epidermidis	+	+	+
coagulase negative staphylococcus	+	+	+
group A streptococcus	+	+	+
group B streptococcus	+	+	+
Strep. mitis	+	+	+
Strep. pneumoniae	+	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.2: 143+3 Gram-positive probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

a



b

Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	+	NT	NT
Enterobac. cloacae	+	+	+
E. coli	+	+	+
E. coli (β-lactamase)	+	NT	NT
K. pneumoniae	+	NT	NT
P. aeruginosa	+	+	+
P. fluorescens	+	+	+
Salmonella Typhimurium	+	+	+
Serr. marcescens	+	NT	NT
Y. enterocolitica	+	+	+

Figure 5.3: Yent2/N6R Gram-negative probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

Staph3 gave strong positive signals for all three staphylococcal species tested, but not with other Gram-positive or Gram-negative species or sterile controls. An example blot and a summary of results obtained for this probe are presented in Table 5.4.

5.3.5 Saur2 - *S. aureus* probe

First published by Kempf (108), this probe proved highly sensitive and specific for its targeted species, *S. aureus*. It did not produce false-positives in the presence of other staphylococci, nor with any other bacterial species. Its specificity was consistent across all sample types. It did not cross-react with human DNA. Sterile PC and RCC controls were also negative. An example blot and a summary of results obtained for this probe are presented in Figure 5.5.

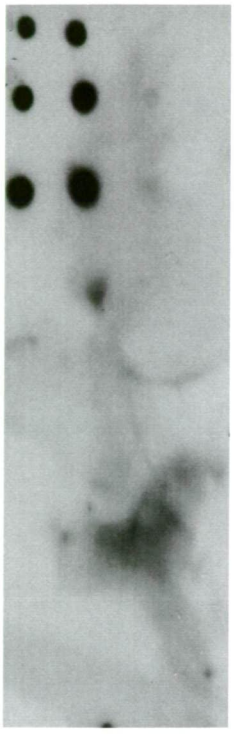
5.3.6 STREP16SR - *Strep. mitis*/*Strep. pneumoniae* probe

Conrads (52) designed this oligonucleotide to be able to detect all *Streptococcus* species when paired with a generic 16S partner. As a probe, it did not detect all *Streptococcus* spp. as hoped, although it did reliably determine the presence of *Strep. mitis* and *Strep. pneumoniae* DNA in samples. Since we were subsequently able to produce probes for the other streptococci of interest (Spy3 for group A and Saga for group B, described in Sections 5.3.7 and 5.3.8, respectively), we continued to test this probe for use as a dual species detector of *Strep. mitis* and *Strep. pneumoniae*. It was found to be highly reliable in the detection of both species in all samples types, without cross-reacting with non-target species or human DNA. PC and RCC controls were negative, as expected. An example blot and a summary of results obtained for this probe are presented in Figure 5.6.

5.3.7 Spy3 - group A streptococcus probe

The Spy3 probe used in this study was based upon the sequence of the Spy probe published by Trebesius (197). The original oligonucleotide sequence had a melting temperature (Tm) of just 48°C and was, therefore, unsuitable for use at our hybridisation temperature of 50°C. Several probes of different Tms were produced, and Spy3 was found to be reliable and specific, and did not cross-react with DNA from other bacterial species or with human DNA in sterile PC and RCC controls.

a



b

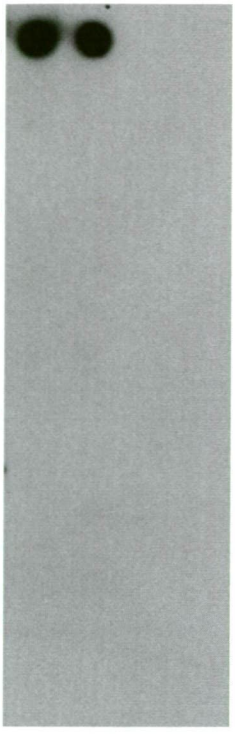
Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	+	+	+
S. epidermidis	+	+	+
coagulase negative staphylococcus	+	+	+
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.4: Staph3 *Staphylococcus* genus probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

a



b

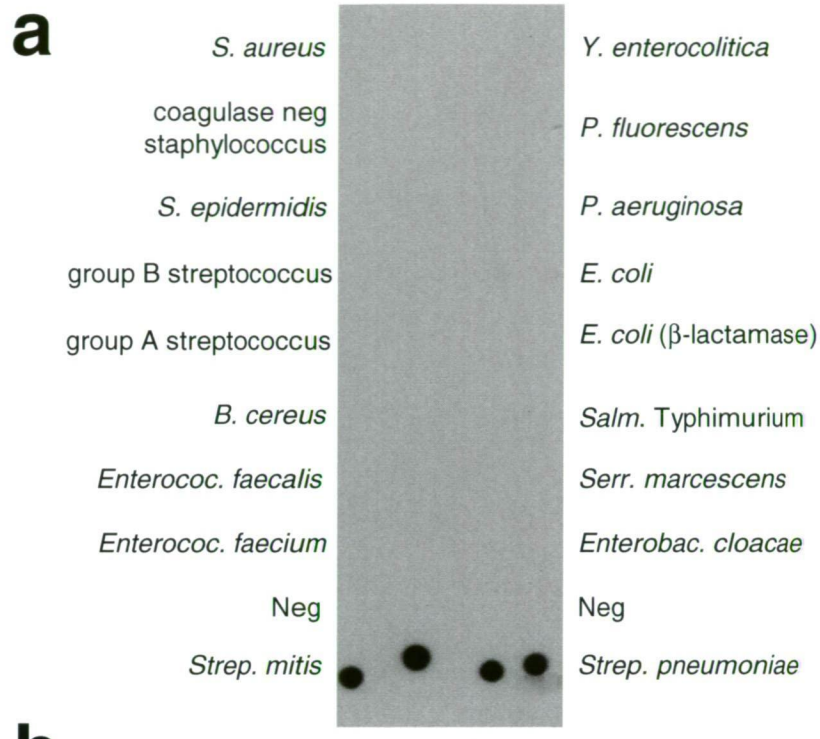
Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	+	+	+
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.5: Saur2 *S. aureus* probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

a



b

Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	+	+	+
Strep. pneumoniae	+	+	+
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.6: STREP16SR *Strep. mitis*/ *Strep. pneumoniae* probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

The sensitivity of probing was equivalent across all sample types, and an example blot and a summary of results obtained for Spy3 probe are presented in Figure 5.7.

5.3.8 Saga - group B streptococcus probe

The Saga probe (also from the laboratory of Trebesius (197)) proved to be specific in the detection of group A streptococcal DNA. This was despite the fact that its predicted Tm was just 2°C above the hybridisation and wash temperatures. It is recommended that all probes are 5 to 10°C above this. No cross-reactions with other DNA or false-positives in our sterile PC and RCC controls were seen, and the sensitivity was not influenced by sample type. An example blot and a summary of results obtained for this probe are presented in Figure 5.8.

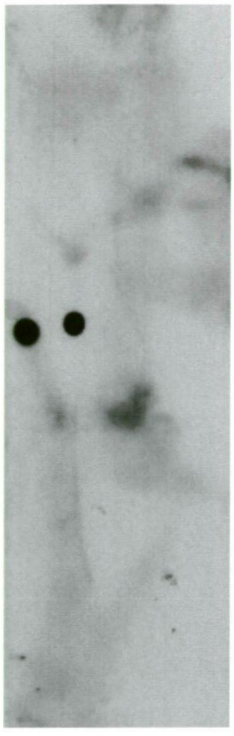
5.3.9 Enc131 - *Enterococcus* genus probe

Several putative *Enterococcus* genus probes were investigated. The most successful of these was Enc131 which was originally used by Behr (17) as a primer for an *Enterococcus* genus PCR. We found it was also highly suitable and specific with all sample types for Southern dot blot, and was able to detect both of our enterococcal test species (*Enterococ. faecalis* and *Enterococ. faecium*), without non-specific detection of DNA from other species or the sterile PC and RCC controls. An example blot and a summary of results obtained for this probe are presented in Figure 5.9.

5.3.10 Bcer1 - *B. cereus* probe

Two *B. cereus* probes were designed for investigation and although both were successful in their targeting, Bcer1 produced the stronger positive signal, and consistently and specifically detected *B. cereus* DNA in all sample types. It did not react with non-target species. All controls were negative as expected. An example blot and a summary of results obtained for this probe are presented in Figure 5.10.

a



b

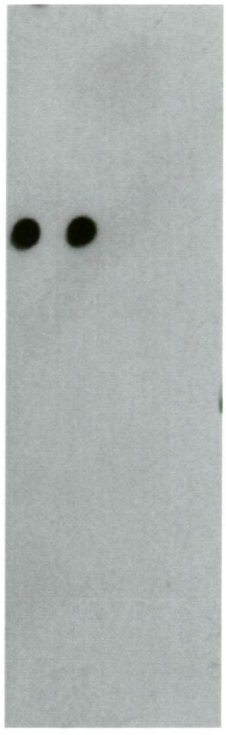
Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	+	+	+
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.7: Spy3 group A streptococcus probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

a



b

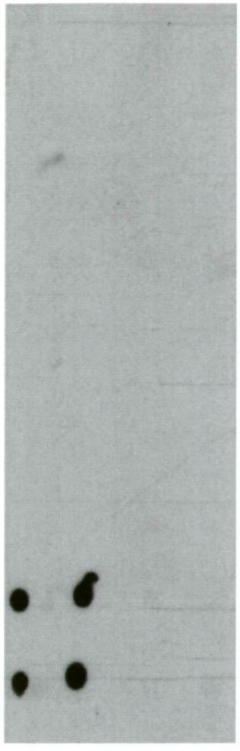
Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	+	+	+
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.8: Saga group B streptococcus probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

a



b

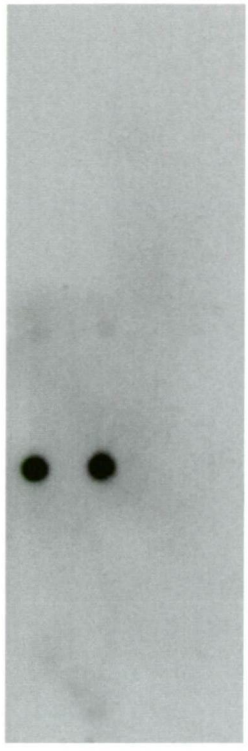
Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	+	+	+
Enterococ. faecium	+	+	+
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.9: Enc131 *Enterococcus* genus probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

a



b

Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	+	+	+
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.10: Bcer *B. cereus* probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

5.3.11 Entero1 - Enteribacteriaceae probe

Probe D (published by Ootsubo) (150) was selected as the basis for the Enteribacteriaceae family investigations. As its Tm was just 43°C, it was redesigned into two new probes. However, neither of these was specific, and they detected both Gram-positive and Gram-negative species (not shown). Hence, a novel probe, Entero1 was designed and successful in detecting all 8 species and strains of Enteribacteriaceae tested, in all sample types. This occurred without cross-reacting with the other Gram-positive or Gram-negative species tested. No false-positive results were seen from our sterile PC and RCC samples. An example blot and a summary of results obtained for this probe are presented in Figure 5.11.

5.3.12 Yent2 - *Y. enterocolitica* probe

After an unsuccessful trial of Yent1 (Trebesius (196)), a novel probe, Yent2, was designed and tested for use against *Y. enterocolitica*. This was shown to be specific in detecting this species in each sample type, and did not produce false-positives in the presence of human DNA or sterile PCs or RCCs. An example blot and a summary of results obtained for this probe are presented in Figure 5.12.

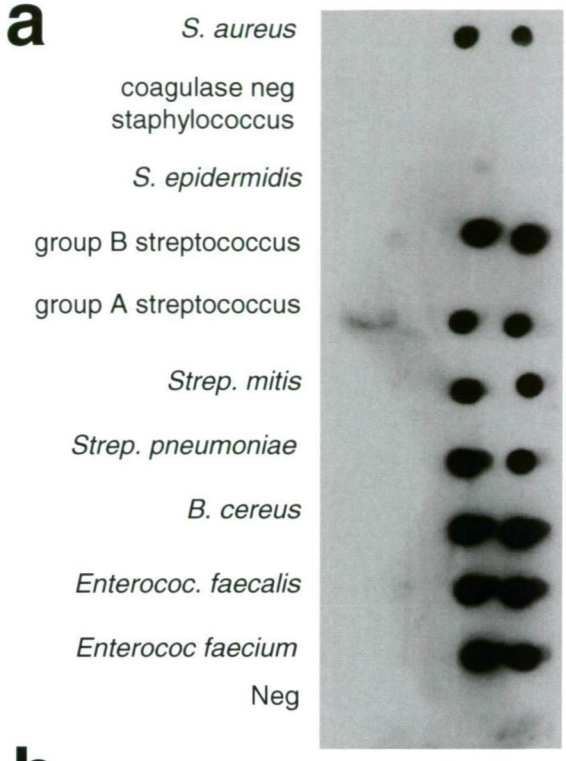
5.3.13 Ppunew5 – *Pseudomonas* genus probe

Ppunew5 was a novel probe designed to selectively detect *P. aeruginosa* and *P. fluorescens*. It successfully did this in all sample types, without false-positive reactions occurring. Human DNA and sterile PCs and RCCs were negative. An example blot and a summary of results obtained for this probe are presented in Figure 5.13.

5.3.14 Paer1 – *P. aeruginosa* probe

Initially, PsaerA was selected for study as a *P. aeruginosa* probe (96), and whilst this probe was able to selectively detect the target species, the positive signal was weak (not shown). A novel probe, Paer1, showed equivalent specificity to the PsaerA probe, but gave a stronger signal in all sample types. This probe did not cross-react with any other bacterial species or human DNA.

a



b

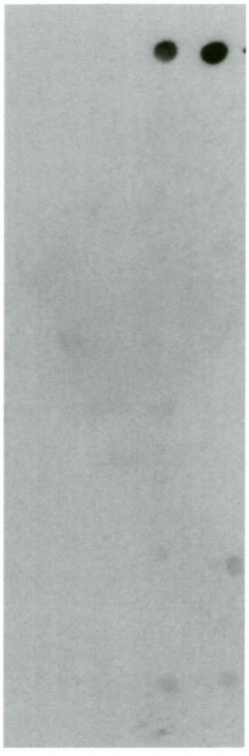
Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	+	NT	NT
Enterobac. cloacae	+	+	+
E. coli	+	+	+
E. coli (β-lactamase)	+	NT	NT
K. pneumoniae	+	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	+	+	+
Serr. marcescens	+	NT	NT
Y. enterocolitica	+	+	+

Figure 5.11: Entero1 Enterobacteriaceae 16S probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

a



b

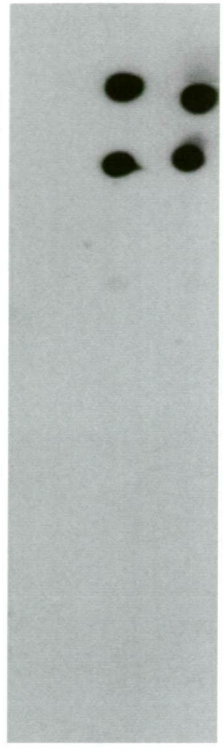
Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	–	–	–
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	+	+	+

Figure 5.12: Yent2 *Y. enterocolitica* probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

a



b

Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	+	+	+
P. fluorescens	+	+	+
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.13: Ppunew5 *Pseudomonas* genus probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe(+, positive result; -, negative result; NT, not tested).

Sterile PC and RCC samples were negative as expected. An example blot and a summary of results obtained for this probe are presented in Figure 5.14.

We had successfully designed or adapted an array of 14 probes, which were able to detect bacterial contamination specifically as designed, using PCR products amplified from pure culture, spiked PC or RCC templates. Frozen growth-kinetic samples from the experiments described in Chapter 3 were subsequently examined using the methods described in this Chapter, to further test the assay.

5.4 Detection and identification of Y. enterocolitica *from frozen samples of blood products using a novel extraction method, large-fragment 16S universal PCR, and Southern dot blot oligoprobing*

The aim of experiments described in this section was to determine whether the success of the extraction method, 16S PCR and Southern dot blot could be replicated with stored frozen samples from our growth kinetic studies. Since we knew the numbers of bacteria in each sample from plate counts conducted at the time of collection, we wanted to determine if we could amplify 16S products from these. As the bacteria had been allowed to grow in the blood products (rather than introduced at known concentrations using washed bacterial cultures), we also wanted to determine if the PCR process or detection was in any way influenced by these different sample types and whether the freezing and long-term (2 years) storage of samples had had any effect. We also wished to determine if the sensitivity of detection was at or below our target of 10^3 CFU/mL, and before product spoilage became evident, as was achieved with spiked products probing.

Selection and preparation of our samples was undertaken as detailed in Section 5.2, and the extracts and PCR products were tested against each of our probes.

5.4.1 Extraction of frozen PC and RCC samples

Once thawed, the consistency of the PC and RCC samples was much more viscous than that of their non-frozen, spiked equivalents. Despite the use of anticoagulants when the sample was first collected, considerable clots were present in most samples (whether the product had spoiled during the kinetic experiments or not). We attempted to homogenise these by manual grinding.

a

b

Species	Washed bacterial cells	Spiked PCs	Spiked RCCs
Gram-positive			
B. cereus	–	–	–
Enterococ. faecalis	–	–	–
Enterococ. faecium	–	–	–
S. aureus	–	–	–
S. epidermidis	–	–	–
coagulase negative staphylococcus	–	–	–
group A streptococcus	–	–	–
group B streptococcus	–	–	–
Strep. mitis	–	–	–
Strep. pneumoniae	–	NT	NT
Gram-negative			
Enterobac. aerogenes	–	NT	NT
Enterobac. cloacae	–	–	–
E. coli	–	–	–
E. coli (β-lactamase)	–	NT	NT
K. pneumoniae	–	NT	NT
P. aeruginosa	+	+	+
P. fluorescens	–	–	–
Salmonella Typhimurium	–	–	–
Serr. marcescens	–	NT	NT
Y. enterocolitica	–	–	–

Figure 5.14: Paer1 *P. aeruginosa* probe results

a) Example blot with washed bacterial cells for PCR template. Duplicate dots.

b) Table summary of accuracy of probe (+, positive result; -, negative result; NT, not tested).

Processing of the 1000 μL frozen samples was the same as for the extraction of the spiked product samples.

5.4.2 1361 bp 16S universal PCR amplification

PCR of samples was conducted in duplicate on two separate occasions. Ten microlitre samples were checked for the presence of amplicons, an example of which is shown in Figure 5.15. Generally, amplification was poor in samples containing 10^3 to 10^7 CFU/mL, and was not evident in samples with fewer than 10^3 CFU/mL. The presence of protein in samples (in the form of a background smear) was also frequently noted. The samples obtained from the experiments (both PC and RCC) with a starting inoculum of 10^2 CFU/mL gave positive PCR results 1 to 2 samplings earlier than specimens inoculated with 10^1 CFU/mL. Amplification did not occur in our negative control PC and RCC extracts.

5.4.3 Confirmation and identification by Southern dot blot

One microlitre PCR samples were spotted in duplicate onto a membrane, and tested against our array of oligonucleotide probes. An example of this is shown in Figure 5.16. RCC samples that had been purified with the QIAquick PCR purification kit were spotted in parallel with non-purified samples, to determine whether this procedure had any effect on the outcome of the Southern dot blots (Figure 5.16b). The results for both starting inocula (10^1 and 10^2) were also compared for any apparent influence over the final result.

5.4.3.1 Specificity of probes

There were two overall groups of probes. One group, we expected to hybridise with *Y. enterocolitica*, while the other group was not expected to hybridise. The specificity of each probe was as found with pure cultures and spiked PCs and RCCs; with the universal 16S (SP16SR), Gram-negative (N6R/Yent2), Enteribacteriaceae (Entero1) and the *Y. enterocolitica* (Yent2) probes all hybridising with the *Y. enterocolitica* amplicon in all sample types, whilst the other probes did not. Subsequently, we divided the two groups of results into separate figures of expected positive probes, (Table 5.1a) and expected negative probes (Table 5.1b).

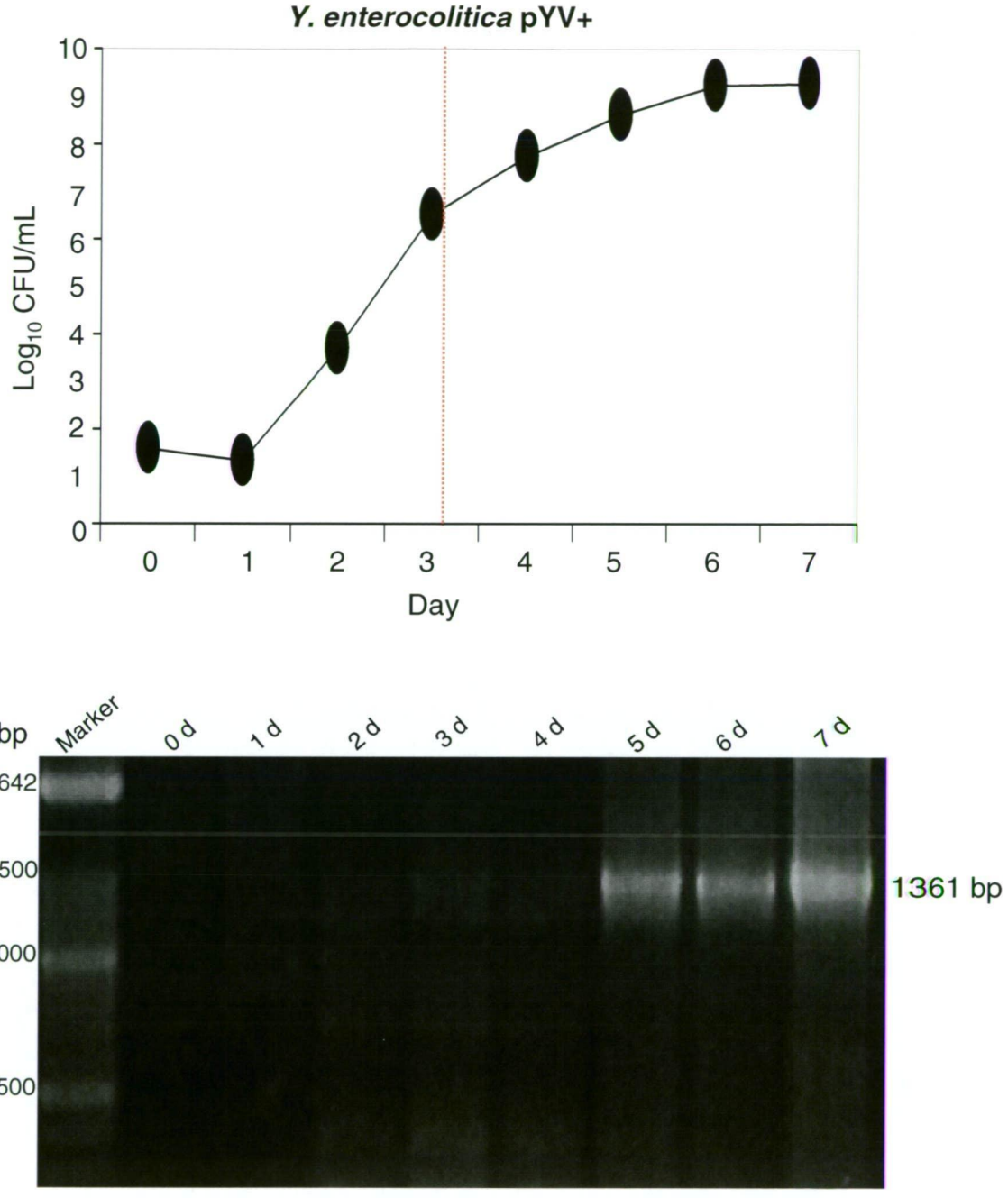


Figure 5.15: Representative16S universal 1361 bp standard PCR using template extracted from growth kinetic platelet concentrates (10^1 CFU/mL inoculum)

Red line on graph indicates when product spoilage occurred.

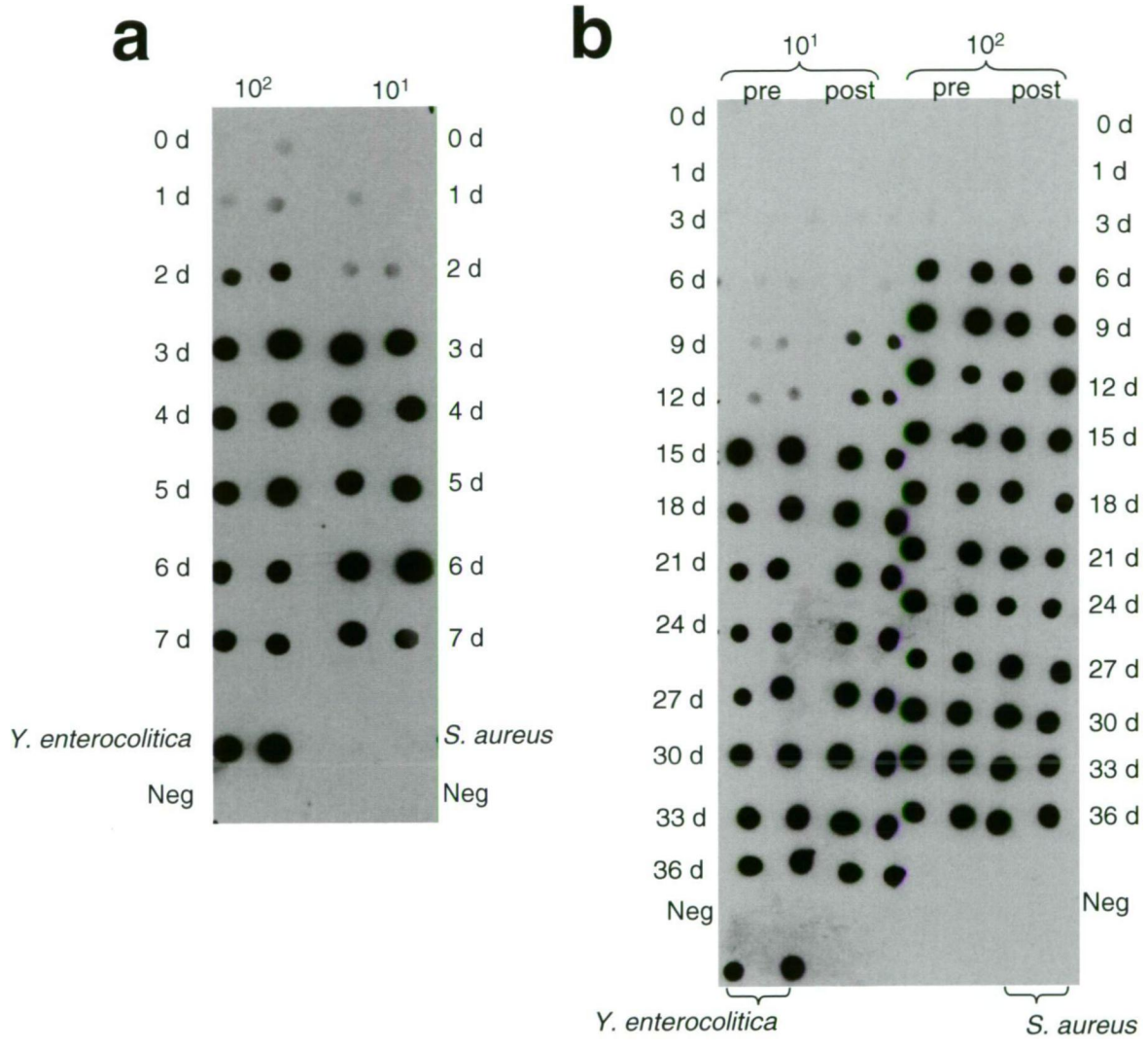


Figure 5.16: Representative frozen blood product sample dot blot results – Yent2 probe

a) PC sample extracts for PCR template

b) RCC sample extracts for PCR template (pre indicates samples not purified with QIAquick PCR purification kit; post indicates samples have been purified with QIAQuick PCR purification kit)

All samples were spotted in duplicate

Table 5.1a: Detection of *Y. enterocolitica* in frozen blood product samples from bacterial growth kinetic studies; targeted probes

	Time-point	Probe								CFU/ mL	
		SP16SR		N6R/Yent2		Entero1		Yent2			
		10^{1} [a]	10^{2}	10^{1}	10^{2}	10^{1}	10^{2}	10^{1}	10^{2}	10^{1}	10^{2}
PC	0 d	–	–	–	–	–	–	–	–	$3.3x10^{1}$	$2.3x10^{2}$
	1 d	–	–	–	–	–	–	–	–	$1.6x10^{1}$	$3.7x10^{3}$
	2 d	–	+	–	+	–	+	–	+	$3.4x10^{3}$	$3.5x10^{7}$
	3 d	+	+	+	+	+	+	+	+	$2.2x10^{6}$	$5.7x10^{8}$
	4 d	+	+	+	+	+	+	+	+	$4.2x10^{7}$	$9.9x10^{8}$
	5 d	+	+	+	+	+	+	+	+	$3.6x10^{8}$	$1.8x10^{9}$
	6 d	+	+	+	+	+	+	+	+	$1.3x10^{9}$	$2.2x10^{9}$
	7 d	+	+	+	+	+	+	+	+	$1.5x10^{9}$	$2.4x10^{9}$
RCC	0 d	–	–	–	–	–	–	–	–	$5.0x10^{0}$	$2.3x10^{1}$
	1 d	–	–	–	–	–	–	–	–	$8.0x10^{0}$	$4.0x10^{1}$
	3 d	–	–	–	–	–	–	–	–	$2.0x10^{2}$	$1.1x10^{3}$
	6 d	–	+	–	+	–	+	–	+	$2.3x10^{5}$	$2.3x10^{5}$
	9 d	+	+	+	+	+	+	+	+	$2.6x10^{8}$	$3.7x10^{8}$
	12 d	+	+	+	+	+	+	+	+	$1.5x10^{9}$	$1.6x10^{9}$
	15 d	+	+	+	+	+	+	+	+	$1.5x10^{9}$	$1.9x10^{9}$
	18 d	+	+	+	+	+	+	+	+	$2.2x10^{9}$	$1.7x10^{9}$
	21 d	+	+	+	+	+	+	+	+	$2.6x10^{9}$	$1.7x10^{9}$
	24 d	+	+	+	+	+	+	+	+	$1.9x10^{9}$	$2.2x10^{9}$
	27 d	+	+	+	+	+	+	+	+	$2.2x10^{9}$	$2.4x10^{9}$
	30 d	+	+	+	+	+	+	+	+	$1.6x10^{9}$	$2.2x10^{9}$
	33 d	+	+	+	+	+	+	+	+	$2.3x10^{9}$	$2.6x10^{9}$
	36 d	+	+	+	+	+	+	+	+	$2.3x10^{9}$	$2.6x10^{9}$

[a], size of original inoculum; +, positive result; -, negative result. Yellow shading indicates where QIAquick purified PCR product from RCC yielded + where previously –. Red shading indicates when spoilage of products became evident.

Table 5.1b: Detection of *Y. enterocolitica* in frozen blood product samples from bacterial growth kinetic studies; non-targeted probes

	Time-point	Probe																				CFU/ mL	
		143+3		Staph3		Saur2		Strep 16SR		Spy3		Saga		Enc131		Bcer1		Ppu new5		Paer1			
		10^1	10^2	10^1	10^2	10^1	10^2	10^1	10^2	10^1	10^2	10^1	10^2	10^1	10^2	10^1	10^2	10^1	10^2	10^1	10^2	10^1	10^2
PC	0 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$3.3x10^1$	$2.3x10^2$
	1 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$1.6x10^1$	$3.7x10^3$
	2 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$3.4x10^3$	$3.5x10^7$
	3 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$2.2x10^6$	$5.7x10^8$
	4 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$4.2x10^7$	$9.9x10^8$
	5 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$3.6x10^8$	$1.8x10^9$
	6 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$1.3x10^9$	$2.2x10^9$
	7 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$1.5x10^9$	$2.4x10^9$
RCC	0 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$5.0x10^0$	$2.3x10^1$
	1 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$8.0x10^0$	$4.0x10^1$
	3 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$2.0x10^2$	$1.1x10^3$
	6 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$2.3x10^5$	$2.3x10^5$
	9 d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$2.6x10^8$	$3.7x10^8$
	12 d	–*	–*	–	–	–	–	–	–	–*	–	–	–	–	–	–	–	–	–	–	–	$1.5x10^9$	$1.6x10^9$
	15 d	–*	–*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$1.5x10^9$	$1.9x10^9$
	18 d	–*	–*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$2.2x10^9$	$1.7x10^9$
	21 d	–*	–*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$2.6x10^9$	$1.7x10^9$
	24 d	–*	–*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$1.9x10^9$	$2.2x10^9$
	27 d	–*	–*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$2.2x10^9$	$2.4x10^9$
	30 d	–*	–*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$1.6x10^9$	$2.2x10^9$
	33 d	–*	–*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$2.3x10^9$	$2.6x10^9$
	36 d	–*	–*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	$2.3x10^9$	$2.6x10^9$

[a], size of original inoculum; *, non-specific detection; +, positive result; -, negative result. Red shading indicates when spoilage of products became evident.

QIAquick PCR cleanup did not have a notable effect on the specificity of the probes, as no background interactions were observed in non-purified or sterile RCC samples (not shown). Whilst the results obtained were as expected, on one occasion, the Gram-positive probe (143+3) appeared to produce positive blots with the RCC extract PCRs (12 d to 36 d). However, repeated experiments with the same probe and samples did not produce this result again (not shown). The 12 d 10^1 RCC sample appeared to give a positive blot with the Spy3 probe, but as with the Gram-positive probe, repeated experiments did not yield this result again. All negative controls (sterile PCs and RCCs) and positive controls (pure cultures of the appropriate species for the probe tested) gave the expected results.

In summary, the specificity of each of the 14 probes tested against our frozen extracts was the same as obtained with pure cultures, and with spiked blood products. None of the Gram-positive or *Pseudomonas* probes were able to hybridise to the *Y. enterocolitica* PCR product, whilst the universal, Gram-negative, Enteribacteriaceae and *Y. enterocolitica* probes all did.

5.4.3.2 Sensitivity of probes

Whilst the specificity of the probes for DNA amplified from the frozen extracts mirrored the results obtained with pure cultures, and with spiked PCs and RCCs, the sensitivity of detection with the frozen samples was much less than that of other sample types tested. Positive signals were obtained in as little as 6 days post inoculation, but the number of CFU/mL in the frozen samples was 2 to 3 orders of magnitude greater than other sample types, and well above our targeted limit of 10^3 CFU/mL. Although QIAquick PCR cleanup had no effect on the specificity of the Southern dot blots, it did produce some effect on the sensitivity, in that the purified RCC 10^1 samples gave positive signals up to 2 sampling time-points earlier than non-purified specimens. However, this observation was not matched by the 10^2 samples. Generally, the sensitivity limitations were between 10^6 and 10^7 CFU/mL for PCs and 10^5 and 10^8 CFU/mL for RCCs. Although this sensitivity was lower than we had anticipated, it may be explained and improved upon in the future in a number ways, as discussed in Section 5.5.

5.5 Discussion

Due to the absence of practicable techniques to screen blood products for bacterial contamination, a range of molecular detection methods were examined. Large fragment 16S universal PCR has been applied to detect bacterial DNA in a range of environmental and clinical settings, and Southern dot blot is commonly used to identify specific sequences in DNA. However, neither of these had been applied together, to detect contaminating bacteria in blood products. When these techniques were combined, they were shown to be highly efficient at detecting bacteria in pure cultures and spiked blood products. Further probes were subsequently developed for this application. Once this method had been evaluated with pure cultures and spiked products, it was further tested on samples obtained from the growth kinetic studies (Chapter 3). This was necessary as bacteria that are allowed to proliferate within stored products may produce substances that could inhibit the detection processes. The extraction of the pure cultures and spiked PCs and RCCs was performed without difficulty, as per Chapter 4. However, the frozen samples from the kinetic studies were highly viscous, and many contained obvious clots. These were found in both spoiled and non-spoiled products (likely to be an artifact of prolonged storage), and were very difficult to disperse evenly throughout the sample prior to the removal of a 1000 μL aliquot for processing. As the lynchpin of the screening method, homogeneity of the sample is essential for the accurate and complete extraction of any bacterial DNA, and its subsequent detection. The viscosity and coagulation of these samples is likely to have influenced the overall sensitivity of the assay, particularly if substantial numbers of bacteria had been bound within the clumps, making the DNA unable to be extracted. Therefore, to determine the true sensitivity of the method, the kinetic studies need to be repeated so that fresh samples can be analysed.

As elucidated in Chapter 4, the sensitivity of the PCR determines the downstream sensitivity of the Southern dot blots. We have shown that PCR of pure cultures and spiked PCs and RCCs allowed us to detect fewer bacteria than our preset limit of 10^3 CFU/mL. However, PCR of the frozen extracts was significantly less successful, with poor amplification occurring in samples containing below 10^7 CFU/mL, making detection possible around the same time as product spoilage became evident. There are several possible explanations for the difference in the

sensitivity of detection obtained with the frozen samples compared to other sample types. As mentioned above, sample clotting may have reduced the actual number of bacteria from which DNA was extracted. Furthermore, it has previously been noted that freezing and long-term storage (more than 2 years in this case) of biological samples may lead to reduced sensitivity of PCR, or reduction of recoverable nucleic acid. Blood is also known to contain many PCR inhibitors (1, 62, 152), the concentration of which may have been increased by freezing or prolonged storage, or they may have been less efficiently removed by the extraction procedure. Interestingly, in some samples the QIAquick purification of RCC PCR products improved the sensitivity of detection by as much as two time-points earlier than their non-purified equivalents. This suggests that in some instances, there may have been inhibitors of DNA hybridisation in the frozen RCC samples. Further investigations should determine if these inhibitors are present only in samples frozen for long periods (thus explaining the need for additional purification of RCC samples in this study), or whether this observation applies to both frozen and non-frozen sample types. The bacteria may also have lysed during storage, causing the breakdown of their nucleic acid that may have undetectable by our large fragment PCR (but may have been detectable with a smaller target). Lastly, the 3 d interval between the 6^{th} and 9^{th} days of RCC storage (and subsequently 3 logs difference between the numbers of CFU recovered) may have also influenced the result. Had samples been taken between these days, detection may have been possible earlier, with a lower number of CFU present. Despite the disappointing sensitivity of PCR in this instance, and given that testing is most likely to occur at the time of sampling, these long-term frozen extracts do not accurately represent the 'real life' testing scenario. It is essential, therefore, that evaluation of the PCR be conducted on truly representative samples, including fresh aliquots taken during the kinetic experiment. We predict that this will significantly improve the sensitivity of detection, as there is no opportunity for the blood to coagulate and 'mask' the true number of organisms in a sample, nor will the freeze-thawing of samples have an influence.

Fourteen oligoprobes were used successfully to detect a range of bacteria in pure cultures and spiked products. These were a collection of published and novel probes, which produced consistently specific results, regardless of the starting

material (Table 5.1a). They were further validated against the frozen extracts, and despite the lower PCR sensitivity, the specificity of the probes was the same as for the other sample types. On two occasions, it appeared that a positive blot had occurred where it was not expected (i.e., the 143+3 probe and the Spy3 probe in Table 5.1b). However, as this result was not reproducible, it is likely that an excess of probe or insufficient blocking of the membrane was responsible for the false-positive result in this instance. Moreover, the strength of the signal from the true positive blots was considerably greater than the signals obtained with the false-positive blots.

In summary, a method by which bacteria can be detected and identified in blood products was developed. Further investigations with freshly collected samples must now occur to address the issue of the sensitivity of the assay. The extraction process is at the centre of the success of this procedure, and with fresh cultures and spiked products samples it was highly effective in yielding pure nucleic acid for PCR. Despite the short sequence of the oligoprobes and the small number of base pair differences in the 16S gene of many species, specific oligoprobes were able detect their targets with a high degree of specificity. It seems likely on balance that this method could be highly suitable for the detection of bacteria in blood products in a centralized testing facility immediately prior to their dispatch, although some additional testing and modification for large-scale use are still required.

CHAPTER SIX

CONCLUDING DISCUSSION

Despite advances in transfusion practices, bacterial contamination of blood products continues to occur, and is currently acknowledged as the greatest infectious risk in transfusion medicine (63, 83, 116, 118, 175). The problem of bacterial contamination is characterised by several features. Bacteria may be introduced in relatively small numbers (1 to 10 CFU/mL) (7, 57, 78, 201) into products during collection (as a result of incomplete donor skin disinfection or asymptomatic bacteraemia) (19, 31, 79, 154), during processing (incomplete tubing seals) (212), or may be present in contaminated equipment (such as non-sterile product bags) (91, 99). Storage conditions, which have been optimised for maximum prolonged preservation of products based on their cellular content, are highly suited for the survival and growth of many bacterial species to high numbers, without outward evidence of their presence. The diverse range of microorganisms involved, has added to the complexity of finding a solution the problem. The frequency at which contamination occurs, does not mirror the frequency of the reports of TTBS due to the acknowledged underreporting of incidents (103, 132). Furthermore, most recipients of blood products are immunocompromised in some way and therefore highly susceptible to infection. Prophylactic antibiotic treatment has not always been able to prevent TTBS in these patients, nor has treatment after the diagnosis of sepsis. Despite the evidence pointing towards the ongoing issue of bacterial contamination and the risks involved to patients, detection of bacterial contamination in blood products manufactured in Australia and many other locales is limited to quality control screening of a small proportion (eg. 1%) of the products. Moreover, bacterial screening of blood products relies on culture-based methods, which although automated, are expensive and can take several days for a definitive result. For these reasons, and the recognised need for more advanced rapid screening measures, the present study was undertaken.

Although previous reports have examined the kinetics of bacteria in plasma rich products (PRP) (7, 31, 47, 73, 160, 201), these differ quite significantly from the buffy coat products used in Europe and Australia. The latter are increasingly preferred for their reduced alloimmunisation effects, but little is known as to how their composition affects bacterial outgrowth following contamination. Through analysis of epidemiological data obtained from the literature and the limited testing

of products at the ARCBS, four distinct groups of bacterial contaminants were identified: psychrophilic, environmental opportunists; mesophilic environmental opportunists; psychrophilic enterics, and skin flora (Aim 1, Chapter 3). Examination of representatives of these groups in stored buffy coat PCs (Figure 3.1) showed that each may proliferate to significant numbers following introduction of an inoculum of as few as 10 CFU/mL bacteria, which is comparable to what may be expected under natural conditions. Furthermore, the ability of these bacteria to proliferate to more than 10^5 CFU/mL (considered a clinical risk dose) (226) within 2 d of storage but without showing physical evidence until day 3 to 4 (10^7 to 10^9 CFU/mL, Figure 3.2) highlights the risk of relying upon spoilage for the identification of contaminated products. When compared with published data pertaining to bacterial growth in PCs obtained from PRP (7, 73, 160, 201), buffy coat PCs were at least equally susceptible to bacterial outgrowth, whilst in some instances (such as *P. fluorescens*) bacterial growth appeared to be more rapid in buffy coat products. This may be due to the reduced plasma content and lower WBC numbers in buffy coat products which may result in less restricted growth of bacteria, especially serum-sensitive strains.

Interestingly, despite cases of sepsis involving *Y. enterocolitica*-contaminated PCs, previous reports found that this species is unable to survive or proliferate in such products (38, 205). Absence of growth has been attributed to the scarcity of free iron in PCs, and the inability of *Y. enterocolitica* to acquire iron from complexed sources such as haemoglobin due to a lack of siderophores. In this study, proliferation of *Y. enterocolitica* was observed in multiple experiments with PCs. The reproducibility of this finding with different PCs make it unlikely that increased free iron in products due to an abnormality in the donor or an increased number of RBCs in the finished blood components, produced this result. We therefore investigated the role of the virulence plasmid, pYV (which confers serum resistance and resistance to phagocytosis) (55), in the survival and proliferation of this species, using a plasmid-less variant of the original parent strain. These studies clearly demonstrated that only the pYV-possessing strain was able to survive and proliferate in PCs (Figure 3.5a), suggesting that *Y. enterocolitica* strains used in previous studies had spontaneously lost the plasmid (a common occurrence) and as a consequence, were susceptible to killing by serum and ingestion by phagocytes. There is also

some evidence that the O3 serotype of *Y. enterocolitica* may have a greater resistance to antimicrobial killing than the O8 serotype (most commonly found in the US, and predominantly used in previous PRP spiking studies). The prevalence of the O3 serotype and its relative resistance to killing may provide a partial explanation for the predominance of this bacterium in reports of TTBS in Australia and New Zealand, although there are no data to show that *Y. enterocolitica* O3 is more resistant to killing in buffy coat PCs than *Y. enterocolitica* O8 (38, 204).

The psychrophilic species freely proliferated in RCCs under refrigerated conditions to reach clinically significant numbers several days before spoilage became evident (Figure 3.3 and 3.4). Contrary to our findings with PCs, the growth of both pYV+ and pYV- strains was similar (Figure 3.5b), indicating that pYV is not necessary for bacterial survival in this product. As the number of WBCs and the amount of natural plasma remaining in PCs and RCCs are comparable, an explanation for the growth of the pYV- strain may be (1) that yop effector genes are not expressed at low temperatures (102) or (2) that any antimicrobial activity in RCCs is slowed or eliminated under refrigeration. This could also explain the lack of clearance of non-proliferating bacteria (such as *S. aureus*) from the RCCs. The persistence of small numbers of bacteria (5 CFU/mL) on its own may be considered unimportant, as these are generally not considered to pose a significant risk to patients. However, there have been instances of TTBS involving *S. aureus* and other non-psychrophilic species that have resulted in patient deaths due to contaminated RCCs (116, 164, 216, 217). This raises the question of what numbers of bacteria are clinically relevant, particularly with immunocompromised recipients. It has also yet to be determined whether the risk in these instances lies with fragmented bacterial remnants (e.g. from bacteria that were introduced into blood products at higher concentrations than 5 CFU/mL and were subsequently killed during storage), or with bacterial proliferation in the transfusion recipient. The survival of *S. aureus* in this instance (whilst reportedly causing TTBS) poses the question, does the clinically significant number of bacteria vary with different bacterial strains? This relates to the production of superantigens, endotoxin and exotoxins (7, 46, 134-136, 140) by different bacterial species, which may be present in excess with few viable microorganisms. Certainly the majority of bacterial species found in blood products

are skin flora (such as coagulase negative staphylococci) or Gram-positive anaerobes (such as *Corynebacterium* and *Propionibacterium* spp.), which despite their linkage with human disease, are often dismissed as insignificant as transfusion risks.

As a bacterial detection method should be useful for all product types, and all groups of bacteria tested were able to proliferate in either or both of PCs and RCCs, all contaminants were considered significant and hence important for detection.

Despite the well known risks posed to patients by bacterially contaminated blood products, it is only in the last two years with the implementation of American Association of Blood Banks standards for the mandatory bacterial screening of PCs, that the issue has begun to be addressed. The ARCBS has recognised the need for new ways to approach the problem of bacterial contamination, and this study (Aim 2) was designed with the intention of developing a novel rapid detection method.

In accordance with the shift of technology towards detection of viral contaminants at the ARCBS using nucleic acid targeting (NAT testing), molecular methods that could be employed using existing infrastructure at the ARCBS were investigated. Preliminary data revealed that that the blood products from which DNA template must be extracted (particularly RCCs) are complex and dense materials, and that the bacteria (particularly the Gram-positive species) were difficult to lyse. Extraction kits recommended for such applications were found to be unsuitable, resulting in DNA yields that were lower than desired, or highly contaminated with proteinaceous residues, or that the protocol was unable to efficiently lyse the bacteria within a convenient timeframe. Furthermore, the target sample volume of 1 mL was unable to be processed due to the considerable amount of cellular debris generated from RCCs. Subsequently. a novel extraction technique was developed that used a combination of bead beating and spin column cleanup to give purified template from both Gram-positive and Gram-negative species in 1 mL PC and RCC samples, which could be detected by PCR using as little as a few fg per sample (at both species-specific and universal 16S PCR levels). The sensitivity of the 1.4 kb 16S universal PCR was equivalent to that obtained with a *Y. enterocolitica*-specific PCR in blood products (70). Furthermore, this PCR proved a powerful tool for discriminating between contaminated and non-contaminated blood products, and was not subject to false-positive results such as those which

occur frequently when smaller-sized 16S DNA fragments are targeted. Discrimination between live and dead bacteria can also be made with this assay, as the nucleic acid of non-viable microorganisms is usually below the target size of this assay, and should therefore not be amplifiable. Although there have been reports of methods to detect bacterial rRNA in blood products directly (without the need of PCR) using oligoprobes (33, 34, 47, 170), these have thus far failed to achieve a sensitivity greater than 10^4 CFU/mL, and the process is too slow for pre-transfusion testing (170). Furthermore, the presence of nucleic acid from non-viable microorganisms or from the blood products themselves (129) and the ubiquitous nature of bacterial nucleic acid may give rise to false-positive results. Another advantage of using the developed method with universal 16S products, is that yet to be identified species that may pose a risk to transfusion recipients in future can be detected with without additional test development.

Comparison of standard PCR and real-time formats for their ability to detect bacterial contamination in blood products using universal targets showed that the standard PCR format provided more sensitive, specific and consistent results than real-time PCR. Whether the sensitivity of the real-time PCR was affected by a high background fluorescence of the SYBR green binding non-specifically to residual protein in the samples (i.e., the template must be of a higher purity for real-time PCR than for standard PCR, due to the optical nature of the detection mechanism and the fact that some protein was visualised in several samples during electrophoresis) has yet to be established, but appears likely. This issue may be resolved in future through further improvement of the template purification process. The use of specific probes rather than non-specific reporters such as SYBR green may also improve the sensitivity and specificity of the assay, although the issue of contaminating environmental DNA (giving rise to false-positive results) must also be addressed before this method can be considered. Until all reagents and plasticware can be provided entirely free of environmental DNA, this will continue to be an issue for universal 16S real-time PCR.

Following amplification of the conserved 1.4 kb 16S target, we showed that identification of the contaminating species was possible using oligoprobes and Southern dot blot hybridisation. Dot blotting has the advantage of obviating gel

transfer (necessary with electrophoresed products), thus speeding up the detection process overall. Using the DIG Easy-Hyb system, the entire process could be completed in just a few hours (plus PCR time), which is within the desired timeframe of testing on the day before or the same day the product is despatched (Chapter 5). The speed of the assay may further be improved with optimisation of the assay to reduce detection time (such as reducing the hybridisation time to 30 min, as suggested by the manufacturer). Furthermore, this study has shown that it may be possible to combine multiple probes (Figure 5.3) to eliminate the need for a separate probe for each target. In smaller blood banks, the minimum requirement of screening lies with a simple yes (contaminated) or no (sterile) answer utilising the universal probe only, perhaps with subsequent identification of the bacterial species by a reference laboratory.

An alternative to the binary yes/no approach with the detection method described may utilise a multiplexed solution of universal, Gram-negative and Gram-positive probes. This might be particularly useful in a diagnostic setting to direct patient therapy. A similar approach was taken by Verax Biomedical (Worcester, MA.) (110), who recently reported an assay that could detect 10^3 to 10^4 CFU/mL of bacteria in PCs using gold-conjugated antibodies targeted to the lipopolysaccharide of Gram-negative bacteria and the lipoteichoic acid of Gram-positive bacteria. However this method and other similar assays (such as Immunetics Inc with their fluorescently–labelled peptidoglycan targeted antibody system) (16) are limited to the detection of bacteria in PCs only. Furthermore, these methods may detect bacterial fragments in the absence of live, whole microorganisms. Although the method developed in his study is comparatively slower than these antibody-based methods, it has the advantage of being applicable to both PCs and RCCs, and preferentially targeting viable bacteria.

The PCR and oligoprobing system developed during the course of this study was highly sensitive and specific with both spiked blood products and pure bacterial extracts, and is therefore likely to be applicable and useful for other sample types. Further validation of the proposed method of detection (Aim 3 of this thesis, the results of which were collated in Chapter 5) involved in a comparison of the spiked blood product results with those obtained for bacterial DNA extracted from frozen

samples collected from the kinetic assays (Chapter 3). However, whilst the specificity of the probes was consistent between sample types, the sensitivity of the PCR using template extracted from the frozen samples was lower than for the spiked samples (Table 5.1). As the screening of blood products is proposed to occur the day before release, it is highly unlikely that the samples for testing will be frozen (certainly not for two years as in this study). Further experiments with freshly collected kinetic samples should be able to resolve these concerns. We anticipate with the repetition of the detection assays using fresh samples, the effects of the freezing (and DNA degradation) will be significantly reduced or eliminated. This hypothesis is supported by reports that freezing interferes with molecular detection of nucleic acid in clinical samples. Even if these fresh samples produce results of reduced sensitivity similar to the frozen samples, the assay may still be useful. As screening is proposed for the day before or the day of product despatch (average of RCCs is 28 d, and of PCs is 4 to 5 d), (12) this may be sufficiently late for these products to contain bacterial numbers above the sensitivity of detection. The findings of Chapter 3 suggest that proliferating bacteria will be between 10^7 and 10^9 CFU/mL in buffy coat blood products after storage for this length of time. Moreover, a highly sensitive assay ($<10^2$ CFU/mL) may result in all blood products being found to contain bacterial DNA (particularly in the case of assays targeting short ribosomal sequences or bacterial fragments, such as LPS or lipoteichoic acid). Would it be necessary to discard all of these? Although these questions are beyond the scope of this study, the current shortage of blood products resulting from short product shelf-life (particularly PCs), and the deferral of many potential donors (e.g., due to malaria risks (69, 144) is well documented.

Another feature of the approach developed here, is the possibility for it to be automated for high-throughput of samples such as required by the blood banking industry. Furthermore, the use of oligoprobes to detect DNA is not unlike the process involved in microarray analysis, which has been proposed as the future of blood testing (155). Although this process is predominantly used for gene expression studies, it has also been used as a bacterial detection method, and may provide a powerful means for high-throughput screening in future (40, 41, 50, 207). Furthermore, PCR conducted directly on the microarray slide may enable the entire

amplification and detection process to be carried out without multiple handling steps (48, 115, 186, 223). Microarray technology is currently comparatively expensive in terms of equipment and reagents. However, as the technology is increasingly adopted, the costs involved should decrease as happened with PCR (155). Furthermore, the ability to process thousands of samples on a single glass slide with small volumes of reagent should also reduce the cost. It has also been proposed to combine blood typing, viral screening and bacterial screening, to confirm the nature of the product contents and ensure its sterility (155). This illustrates a strength of the approach used in this detection method, namely its versatility in terms of sample types and target species detected, with potential for adaptation to other platforms such as microarray.

In summary, the 'zero risk' approach taken to blood banking has increased awareness of the problem of bacterial contamination of blood products, and emphasised the need for ways to detect this rapidly. This study found that a diverse array of bacterial contaminants found in blood products can survive and proliferate in both buffy coat PCs and RCCs. Further to this, detection of these bacterial pathogens can be achieved rapidly and sensitively by targeting conserved 16S rDNA sequences, with further identification provided by hybridisation with oligoprobes. As yet unidentified bacterial contaminants may also be detected in this way. Although further validation of this technique is required, this approach may also be useful beyond the transfusion setting, in diagnostic medicine, or quality control of food or other substances. Furthermore, there is potential for using this detection method as the basis for further novel detection assays, such as microarray screening which should allow more samples to be processed in a shorter time-period, and should become increasingly attractive as the cost of the technology decreases.

REFERENCES

1. **Abu Al-Soud, W., and P. Radstrom.** 1998. Capacity of nine thermostable DNA polymerases To mediate DNA amplification in the presence of PCR-inhibiting samples. Appl. Environ. Microbiol. **64:**3748-53.
2. **Allain, J. P.** 2000. Genomic screening for blood-borne viruses in transfusion settings. Clin. Lab. Haematol. **22:**1-10.
3. **American Association of Blood Banks.** 2002. AABB standards for blood banks and transfusion services., 22nd ed. American Association of Blood Banks, Bethesda, MA.
4. **American Association of Blood Banks.** 2003. Further guidance on methods to detect bacterial contamination of platelet components. Association Bulletin 03a-12. American Association of Blood Banks, Bethesda, MA.
5. **Anderson, K. C., M. A. Lew, B. C. Gorgone, J. Martel, C. B. Leamy, and B. Sullivan.** 1986. Transfusion-related sepsis after prolonged platelet storage Am. J. Med. **81:**405-11.
6. **Anstall, H. B., R. C. Blaylock, and C. M. Craven.** 1993. Managing hazards in the transfusion service. ASCP Press, Chicago.
7. **Arduino, M. J., L. A. Bland, M. A. Tipple, S. M. Aguero, M. S. Favero, and W. R. Jarvis.** 1989. Growth and endotoxin production of *Yersinia enterocolitica* and *Enterobacter agglomerans* in packed erythrocytes. J. Clin. Microbiol. **27:**1483-5.
8. **Arnold, L. J., Jr., P. W. Hammond, W. A. Wiese, and N. C. Nelson.** 1989. Assay formats involving acridinium-ester-labelled DNA probes. Clin. Chem. **35:**1588-94.
9. **Astiz, M. E., and E. C. Rackow.** 1998. Septic shock. Lancet. **351:**1501-5.
10. **AuBuchon, J. P., C. Pickard, and L. Herschel.** 1995. Sterility of plastic tubing welds in components stored at room temperature. Transfusion. **35:**303-7.
11. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** Current Protocols in Molecular Biology. Greene Pub. Associates and Wiley-Interscience, New York.

12. **Baleriola-Lucas, C.** 2001. Bacterial Contamination data. Australian Red Cross Blood Service.

13. **Balligand, G., Y. Laroche, and G. Cornelis.** 1985. Genetic analysis of virulence plasmid from a serogroup 9 *Yersinia enterocolitica* strain: role of outer membrane protein P1 in resistance to human serum and autoagglutination. Infect. Immun. **48:**782-6.

14. **Barrett, B. B., J. W. Andersen, and K. C. Anderson.** 1993. Strategies for the avoidance of bacterial contamination of blood components. Transfusion. **33:**228-33.

15. **Barski, P., L. Piechowicz, J. Galinski, and J. Kur.** 1996. Rapid assay for detection of methicillin-resistant *Staphylococcus aureus* using multiplex PCR. Mol. Cell. Probes. **10:**471-5.

16. **Beausang, L. A., and A. Levin.** 2003. Development of a rapid assay for the detection of bacteria in platelet units. Transfusion. **43S:**20A.

17. **Behr, T., C. Koob, M. Schedl, A. Mehlen, H. Meier, D. Knopp, E. Frahm, U. Obst, K. Schleifer, R. Niessner, and W. Ludwig.** 2000. A nested array of rRNA targeted probes for the detection and identification of enterococci by reverse hybridization. Syst. Appl. Microbiol. **23:**563-72.

18. **Bertolini, F., and S. Murphy.** 1994. A multicenter evaluation of reproducibility of swirling in platelet concentrates. Biomedical Excellence for Safer Transfusion (BEST) Working Party of the International Society of Blood Transfusion. Transfusion. **34:**796-801.

19. **Bhanji, S., B. Williams, B. Sheller, T. Elwood, and L. Mancl.** 2002. Transient bacteremia induced by toothbrushing a comparison of the Sonicare toothbrush with a conventional toothbrush. Pediatr. Dent. **24:**295-9.

20. **Blachman, M. A., A. Ali, and P. Lyn.** 1997. Bacterial surveillance of platelet concentrates: Quantitation of bacterial load. Transfusion. **37:**74S.

21. **Black, R. E., R. J. Jackson, T. Tsai, M. Medvesky, M. Shayegani, J. C. Feeley, K. I. MacLeod, and A. M. Wakelee.** 1978. Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. N. Engl. J. Med. **298:**76-9.

22. **Blajchman, M. A.** 1997. Allogeneic blood transfusions, immunomodulation, and postoperative bacterial infection: do we have the answers yet? Transfusion. **37:**121-5.

23. **Blajchman, M. A.** 2004. Bacterial contamination of cellular blood components: risks, sources and control. Vox. Sang. **87:**98-103.

24. **Blajchman, M. A.** 2002. Incidence and significance of the bacterial contamination of blood components. Dev. Biol. (Basel). **108:**59-67.

25. **Blajchman, M. A.** 1994. Transfusion-associated bacterial sepsis: the phoenix rises yet again Transfusion. **34:**940-2.

26. **Blajchman, M. A., A. M. Ali, and H. L. Richardson.** 1994. Bacterial contamination of cellular blood components. Vox. Sang. **67 Suppl 3:**25-33.

27. **Blajchman, M. A., P. Lyn, and E. Rosenberg.** 1996. Bacterial surveillance of platelet concentrates: comparison of day 1 versus day 3 bacterial cultures. Blood. **88:**286.

28. **Borden, C. W., and W. H. Hall.** 1951. Fatal transfusion reactions from massive bacterial contamination of blood. N. Engl. J. Med. **245:**760-5.

29. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935-43.

30. **Borun, M., A. Sajduda, I. Pawlowska, J. J. McFadden, and J. Dziadek.** 2001. Detection of *Mycobacterium tuberculosis* in clinical samples using insertion sequences IS6110 and IS990. Tuberculosis (Edinb). **81:**271-8.

31. **Braine, H. G., T. S. Kickler, P. Charache, P. M. Ness, J. Davis, C. Reichart, and A. K. Fuller.** 1986. Bacterial sepsis secondary to platelet transfusion: an adverse effect of extended storage at room temperature. Transfusion. **26:**391-3.

32. **Braude, A. I., J. P. Sanford, J. E. Bartlett, and O. T. Mallery, Jr.** 1952. Effects and clinical significance of bacterial contaminants in transfused blood. J. Lab. Clin. Med. **39:**902-16.

33. **Brecher, M. E., G. Boothe, and A. Kerr.** 1993. The use of a chemiluminescence-linked universal bacterial ribosomal RNA gene probe and blood gas analysis for the rapid detection of bacterial contamination in white cell-reduced and nonreduced platelets. Transfusion. **33:**450-7.

34. **Brecher, M. E., J. J. Hogan, G. Boothe, A. Kerr, L. McClannan, M. R. Jacobs, R. Yomtovian, V. Chongokolwatana, G. Tegtmeier, S. Henderson.** 1994. Platelet bacterial contamination and the use of a chemiluminescence-linked universal bacterial ribosomal RNA gene probe Transfusion. **34:**750-5.

35. **Brecher, ME., and S. N. Hay.** 2005. Bacterial contamination of blood components. Clin. Microbiol. Rev. **18**: 195-204.

36. **Bruneau, C., P. Perez, M. Chassaigne, P. Allouch, A. Audurier, C. Gulian, G. Janus, G. Boulard, P. De Micco, L. R. Salmi, and L. Noel.** 2001. Efficacy of a new collection procedure for preventing bacterial contamination of whole-blood donations. Transfusion. **41:**74-81.

37. **Buchholz, D. H., J. P. AuBuchon, E. L. Snyder, R. Kandler, S. Edberg, V. Piscitelli, C. Pickard, and P. Napychank.** 1992. Removal of *Yersinia enterocolitica* from AS-1 red cells. Transfusion. **32:**667-72.

38. **Buchholz, D. H., J. P. AuBuchon, E. L. Snyder, R. Kandler, V. Piscitelli, C. Pickard, P. Napychank, and S. Edberg.** 1994. Effects of white cell reduction on the resistance of blood components to bacterial multiplication. Transfusion. **34:**852-7.

39. **Busch, M. P., S. H. Kleinman, and G. J. Nemo.** 2003. Current and emerging infectious risks of blood transfusion. J. Am. Med. Soc. **289:**952-962.

40. **Call, D. R., M. K. Borucki, and F. J. Loge** 2003. Detection of bacterial pathogens in environmental samples using DNA microarrays J Microbiol Methods. **53:**235-43.

41. **Call, D. R., F. J. Brockman, and D. P. Chandler.** 2001. Detecting and genotyping Escherichia coli O157:H7 using multiplexed PCR and nucleic acid microarrays. Int. J. Food. Microbiol. **67:**71-80.

42. **Carroll, N. M., P. Adamson, and N. Okhravi.** 1999. Elimination of bacterial DNA from *Taq* DNA polymerases by restriction endonuclease digestion. J. Clin. Microbiol. **37:**3402-4.

43. **Carroll, N. M., E. E. Jaeger, S. Choudhury, A. A. Dunlop, M. M. Matheson, P. Adamson, N. Okhravi, and S. Lightman.** 2000. Detection of and discrimination between gram-positive and gram-negative bacteria in intraocular samples by using nested PCR. J. Clin. Microbiol. **38:**1753-7.

44. **Casewell, M. W., N. G. Slater, and J. E. Cooper.** 1981. Operating theatre water-baths as a cause of pseudomonas septicaemia. J. Hosp. Infect. **2:**237-47.

45. **Castro, E. M., J. L. Bueno, R. Gonzalez, and L. Barea.** 1999. Implementation of an automated bacterial culture of platelet products in the blood bank routine. Transfusion. **39:**75S.

46. **Centres for Disease Control.** 1991. From the Centers for Disease Control. *Yersinia enterocolitica* bacteremia and endotoxin shock associated with red blood cell transfusions-United States. JAMA. **265:**2174-5.

47. **Chaney, R., J. Rider, and D. Pamphilon.** 1999. Direct detection of bacteria in cellular blood products using bacterial ribosomal RNA-directed probes coupled to electrochemiluminescence. Transfus. Med. **9:**177-88.

48. **Cheng, J., M. A. Shoffner, G. E. Hvichia, L. J. Kricka, and P. Wilding.** 1996. Chip PCR. II. Investigation of different PCR amplification systems in microbabricated silicon-glass chips. Nucleic. Acids. Res. **24:**380-5.

49. **Chiu, E. K., K. Y. Yuen, A. K. Lie, R. Liang, Y. L. Lau, A. C. Lee, Y. L. Kwong, S. Wong, M. H. Ng, and T. K. Chan.** 1994. A prospective study of symptomatic bacteremia following platelet transfusion and of its management. Transfusion. **34:**950-4.

50. **Chizhikov, V., A. Rasooly, K. Chumakov, and D. D. Levy.** 2001. Microarray analysis of microbial virulence factors. Appl. Environ. Microbiol. **67:**3258-63.

52. **Chongokolwatana, V., M. Morgan, J. C. Feagin, M. R. Jacobs, and R. Yomotovian.** 1993. Comparison of microscopy and a bacterial DNA probe for detecting bacterially contaminated platelets. Transfusion. **33S:**50S, 191a.

52. **Conrads, G., S. E. Gharbia, K. Gulabivala, F. Lampert, and H. N. Shah.** 1997. The use of a 16s rDNA directed PCR for the detection of endodontopathogenic bacteria. J. Endod. **23:**433-8.

53. **Contreras, M.** (ed.). 1998. ABC of Transfusion, 3rd ed. BMJ Books, London.

54. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, E. B. Kaczmarski, and A. J. Fox.** 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J. Clin. Microbiol. **38:**1747-52.

55. **Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory, and I. Stainier.** 1998. The virulence plasmid of *Yersinia*, an antihost genome. Microbiol. Mol. Biol. Rev. **62:**1315-52.

56. **Council of Europe.** 2003. Guide to the preparation, use and quality assurance of blood components, 10th ed.

57. **Cover, T. L., and R. C. Aber** 1989. *Yersinia enterocolitica.* N. Engl. J. Med. **321:**16-24.

58. **Cox, D. L., P. Chang, A. W. McDowall, and J. D. Radolf.** 1992. The outer membrane, not a coat of host proteins, limits antigenicity of virulent *Treponema pallidum.* Infect. Immun. **60:**1076-83.

59. **Cumming, P. D., E. L. Wallace, J. B. Schorr, and R. Y. Dodd.** 1989. Exposure of patients to human immunodeficiency virus through the transfusion of blood components that test antibody-negative. N. Engl. J. Med. **321:**941-6.

60. **Cursons, R. T., E. Jeyerajah, and J. W. Sleigh.** 1999. The use of polymerase chain reaction to detect septicemia in critically ill patients. Crit. Care. Med. **27:**937-40.

61. **Daly, P., T. Collier, and S. Doyle.** 2002. PCR-ELISA detection of *Escherichia coli* in milk. Lett. Appl. Microbiol. **34:**222-6.

62. **de Franchis, R., N. C. Cross, N. S. Foulkes, and T. M. Cox.** 1988. A potent inhibitor of *Taq* polymerase copurifies with human genomic DNA. Nucleic. Acids. Res. **16:**10355.

63. **Debeir, J., L. Noel, J. Aullen, C. Frette, F. Sari, M. P. Mai, and A. Cosson.** 1999. The French haemovigilance system. Vox. Sang. **77:**77-81.

64. **Dodd, R. Y., E. P. t. Notari, and S. L. Stramer.** 2002. Current prevalence and incidence of infectious disease markers and estimated window-period risk in the American Red Cross blood donor population. Transfusion **42:**975-9.

65. **Dzieczkowski, J. S., B. B. Barrett, D. Nester, M. Campbell, J. Cook, M. Sugrue, J. W. Andersen, and K. C. Anderson.** 1995. Characterization of reactions after exclusive transfusion of white cell-reduced cellular blood components. Transfusion. **35:**20-5.

66. **Dzik, W.** 1995. Use of leukodepletion filters for the removal of bacteria. Immunol. Invest. **24:**95-115.

67. **EcEntegtart, M. G.** 1956. Dangerous contaminants in stored blood. Lancet. **271:**909-911.

68. **Elrick, J.** 1988. A case study - fatal gram-negative shock following blood transfusions. Yersinia News (Melbourne). **5:**1-2.

69. **Farrugia, A.** 2004. The mantra of blood safety: time for a new tune? Vox. Sang. **86:**1-7.

70. **Feng, P., S. P. Keasler, and W. E. Hill.** 1992. Direct identification of *Yersinia enterocolitica* in blood by polymerase chain reaction amplification. Transfusion. **32:**850-4.

71. **Fijnvandraat, K., B. Derkx, M. Peters, R. Bijlmer, A. Sturk, M. H. Prins, S. J. van Deventer, and J. W. ten Cate.** 1995. Coagulation activation and tissue necrosis in meningococcal septic shock: severely reduced protein C levels predict a high mortality. Thromb. Haemost. **73:**15-20.

71. **Fourrier, F., C. Chopin, J. Goudemand, S. Hendrycx, C. Caron, A. Rime, A. Marey, and P. Lestavel.** 1992. Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared patterns of antithrombin III, protein C, and protein S deficiencies. Chest. **101:**816-23.

73. **Franzin, L., and P. Gioannini.** 1992. Growth of *Yersinia* species in artificially contaminated blood bags. Transfusion. **32:**673-6.

74. **Friedman, G., E. Silva, and J. L. Vincent.** 1998. Has the mortality of septic shock changed with time. Crit. Care. Med. **26:**2078-86.

75. **Garnham, P. C. C.** 1966. Malaria parasites and other haemosporidia. Blackwell Scientific, Oxford.

76. **Gibb, A. P., K. M. Martin, G. A. Davidson, B. Walker, and W. G. Murphy.** 1994. Modeling the growth of *Yersinia enterocolitica* in donated blood. Transfusion. **34:**304-10.

77. **Gibson, T., and W. Norris.** 1958. Skin fragments removed by injection needles. Lancet. **2:**983-5.

78. **Goldman, M.** 2004. Bacterial contamination of platelet concentrates: where are we today? Vox. Sang. **87:**90-2.

79. **Goldman, M., and M. A. Blajchman.** 1991. Blood product-associated bacterial sepsis. Transfus. Med. Rev. **5:**73-83.

80. **Goldman, M., G. Roy, N. Frechette, F. Decary, L. Massicotte, and G. Delage.** 1997. Evaluation of donor skin disinfection methods. Transfusion. **37:**309-12.

81 **Goodnough, L. T., A. Shander, and M. E. Brecher.** 2003. Transfusion medicine: looking to the future. Lancet. **361:** 161-9.

82. **Gottlieb, T.** 1993. Hazards of bacterial contamination of blood products. Anaesth. Intensive. Care. **21:**20-3.

83. **Gozzard, D.** 2000. Serious Hazards of Transfusion, (SHOT). [Online.] http:// www.shot.demon.co.uk/ toc.htm.

84. **Grant, T., V. Bennett-Wood, and R. M. Robins-Browne.** 1999. Characterization of the interaction between *Yersinia enterocolitica* biotype 1A and phagocytes and epithelial cells in vitro. Infect. Immun. **67:**4367-75.

85. **Grant, T., V. Bennett-Wood, and R. M. Robins-Browne.** 1998. Identification of virulence-associated characteristics in clinical isolates of *Yersinia enterocolitica* lacking classical virulence markers. Infect. Immun. **66:**1113-20.

86. **Greene, J. N., J. T. Sinnot, J. A. Wychoff, L. Carter, and R. L. Sandin.** 1995. Gram staining of blood. Infect. Med. **12:**165-70.

87. **Greene, R. C.** 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. **28:**1942-1946.

88. **Greisen, K., M. Loeffelholz, A. Purohit, and D. Leong.** 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J. Clin. Microbiol. **32:**335-51.

89. **Grisold, A. J., E. Leitner, G. Muhlbauer, E. Marth, and H. H. Kessler.** 2002. Detection of methicillin-resistant *Staphylococcus aureus* and simultaneous confirmation by automated nucleic acid extraction and real-time PCR. J. Clin. Microbiol. **40:**2392-7.

90. **Heal, J. M., M. E. Jones, J. Forey, A. Chaudhry, and R. L. Stricof.** 1987. Fatal *Salmonella* septicemia after platelet transfusion. Transfusion. **27:**2-5.

91. **Heltberg, O., F. Skov, P. Gerner-Smidt, H. J. Kolmos, E. Dybkjaer, E. Gutschik, D. Jerne, O. B. Jepsen, M. Weischer, and W. Frederiksen.** 1993. Nosocomial epidemic of *Serratia marcescens* septicemia ascribed to contaminated blood transfusion bags. Transfusion. **33:**221-7.

92. **Hendolin, P. H., A. Markkanen, J. Ylikoski, and J. J. Wahlfors.** 1997. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. J. Clin. Microbiol. **35:**2854-8.

93. **Hewlett, I. K., and J. S. Epstein.** 1997. Food and Drug Administration conference on the feasibility of genetic technology to close the HIV window in donor screening. Transfusion. **37:**346-51.

94. **Hilali, F., P. Saulnier, E. Chachaty, and A. Andremont.** 1997. Decontamination of polymerase chain reaction reagents for detection of low concentrations of 16S rRNA genes. Mol. Biotechnol. **7:**207-16.

95. **Hillmore, S.** 2000. Malaria. Random House, Sydney.

96. **Hogardt, M., K. Trebesius, A. M. Geiger, M. Hornef, J. Rosenecker, and J. Heesemann.** 2000. Specific and rapid detection by fluorescent in situ hybridization of bacteria in clinical samples obtained from cystic fibrosis patients. J. Clin. Microbiol. **38:**818-25.

97. **Hogman, C. F.** 1996. Bacterial transmission: a continuing challenge. Transfus. Microbiol. **24:**1.

98. **Hogman, C. F., and L. Engstrand.** 1996. Factors affecting growth of *Yersinia enterocolitica* in cellular blood products. Transfus. Med. Rev. **10:**259-75.

99. **Hogman, C. F., H. Fritz, and L. Sandberg.** 1993. Posttransfusion *Serratia marcescens* septicemia. Transfusion. **33:**189-91.

100. **Hogman, C. F., J. Gong, L. Eriksson, A. Hambraeus, and C. S. Johansson.** 1991. White cells protect donor blood against bacterial contamination. Transfusion. **31:**620-6.

101. **Horsburgh, C. R., Jr., C. Y. Ou, J. Jason, S. D. Holmberg, I. M. Longini, Jr., C. Schable, K. H. Mayer, A. R. Lifson, G. Schochetman, and J. W. Ward.** 1989. Duration of human immunodeficiency virus infection before detection of antibody. Lancet. **2:**637-40.

102. **Jacobs, K. A., R. Rudersdorf, S. D. Neill, J. P. Dougherty, E. L. Brown, and E. F. Fritsch.** 1988. The thermal stability of oligonucleotide duplexes is sequence independent in tetraalkylammonium salt solutions: application to identifying recombinant DNA clones. Nucleic. Acids. Res. **16:**4637-50.

103. **Jacobs, M. R., E. Palavecino, and R. Yomtovian.** 2001. Don't bug me: the problem of bacterial contamination of blood components--challenges and solutions. Transfusion. **41:**1331-4.

104. **Jaffe, R. I., J. D. Lane, and C. W. Bates.** 2001. Real-time identification of Pseudomonas aeruginosa direct from clinical samples using a rapid extraction method and polymerase chain reaction (PCR). J. Clin. Lab. Anal. **15:**131-7.

105. **Janot, C., M. E. Briquel, F. Streiff, and J. C. Burdin.** 1989. Infectious complications due to transfusion acquired *Yersinia enterocolitica.* Transfusion. **29:**372-3.

106. **Jorgensen, J. H., G. V. Doern, M. J. Ferraro, C. C. Knapp, J. M. Swenson, and J. A. Washington.** 1992. Multicenter evaluation of the use of *Haemophilus* test medium for broth microdilution antimicrobial susceptibility testing of *Streptococcus pneumoniae* and development of quality control limits. J. Clin. Microbiol. **30:**961-6.

107. **Kaufhold, A., A. Podbielski, G. Baumgarten, M. Blokpoel, J. Top, and L. Schouls.** 1994. Rapid typing of group A streptococci by the use of DNA amplification and non-radioactive allele-specific oligonucleotide probes. FEMS. Microbiol. Lett. **119:**19-25.

108. **Kempf, V. A., K. Trebesius, and I. B. Autenrieth.** 2000. Rapid identification of pathogens in blood. Ann. Intern. Med. **132:**330-1.

109. **Kendrick, C. J., B. Baker, A. J. Morris, and P. W. O'Toole.** 2001. Identification of *Yersinia*-infected blood donors by anti-Yop IgA immunoassay. Transfusion. **41:**1365-72.

110. **Kenny, F., C. Woodbury, M. Pelak, J. Weinberg, and J. A. Hall.** 2003. Development of a rapid test for detection of bacterial contamination in platelet concentrates. Transfusion. **43S:**48A.

111. **Kiely, P.** 2004. Virus Serology, ARCBS. Viral transmission data.

112. **Kim, D. M., M. E. Brecher, L. A. Bland, T. J. Estes, R. A. Carmen, and E. J. Nelson.** 1992. Visual identification of bacterially contaminated red cells. Transfusion. **32:**221-5.

113. **Kim, D. M., M. E. Brecher, L. A. Bland, T. J. Estes, S. K. McAllister, S. M. Aguero, R. A. Carmen, and E. J. Nelson.** 1992. Prestorage removal of *Yersinia enterocolitica* from red cells with white cell-reduction filters. Transfusion. **32:**658-62.

114. **Klausegger, A., M. Hell, A. Berger, K. Zinober, S. Baier, N. Jones, W. Sperl, and B. Kofler.** 1999. Gram type-specific broad-range PCR amplification for rapid detection of 62 pathogenic bacteria. J. Clin. Microbiol. **37:**464-6.

115. **Kopp, M. U., A. J. Mello, and A. Manz.** 1998. Chemical amplification: continuous-flow PCR on a chip. Science. **280:**1046-8.

116. **Kuehnert, M. J., V. R. Roth, N. R. Haley, K. R. Gregory, K. V. Elder, G. B. Schreiber, M. J. Arduino, S. C. Holt, L. A. Carson, S. N. Banerjee, and W. R. Jarvis.** 2001. Transfusion-transmitted bacterial infection in the United States, 1998 through 2000. Transfusion. **41:**1493-9.

117. **Lane, D. J.** 1991. 16S/23S rRNA sequencing., p. 115-175. *In* E. a. G. Stackebrandt, M (ed.), Nucleic Acid Technology in Bacterial Systematics. John Wiley and Sons, New York.

118. **Lee, J.** 1999. Review of Heidelberg Symposium and FDA fatality reports. Presented at the FDA/CBER Bacterial Contamination of Platelets Workshop, MD, Bethesda, United States Food and Drug Administration, September 1999. United States Food and Drug Administration. [Online.] http://www.fda.gov/cber/min/bact092499.pdf.

119. **Leiby, D. A., K. L. Kerr, J. M. Campos, and R. Y. Dodd.** 1997. A retrospective analysis of microbial contaminants in outdated random-donor platelets from multiple sites. Transfusion. **37:**259-63.

120. **Levi, M., E. de Jonge, T. van der Poll, and H. ten Cate.** 1999. Disseminated intravascular coagulation. Thromb. Haemost. **82:**695-705.

121. **Levi, M., H. ten Cate, T. van der Poll, and S. J. van Deventer.** 1993. Pathogenesis of disseminated intravascular coagulation in sepsis. JAMA. **270:**975-9.

122. **Liu, H. W., K. Y. Yuen, T. S. Cheng, K. B. Lee, E. K. Chua, P. L. Ho, and C. K. Lin.** 1999. Reduction of platelet transfusion- associated sepsis by short-term bacterial culture. Vox. Sang. **77:**1-5.

123. **Lovric, V. A.** 1988. Another Australian case of fatal septic shock following blood transfusion. Yersinia News (Melbourne). **7:**1.

124. **Lu, J. J., C. L. Perng, S. Y. Lee, and C. C. Wan.** 2000. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. J. Clin. Microbiol. **38:**2076-80.

125. **Maki, D. G., M. Ringer, and C. J. Alvarado.** 1991. Prospective randomised trial of povidone-iodine, alcohol, and chlorhexidine for prevention of infection associated with central venous and arterial catheters. Lancet. **338:**339-43.

126. **Mariani, B. D., D. S. Martin, M. J. Levine, R. E. Booth, Jr., and R. S. Tuan.** 1996. The Coventry Award. Polymerase chain reaction detection of bacterial infection in total knee arthroplasty. Clin. Orthop. **35:**11-22.

127. **Martineau, F., F. J. Picard, D. Ke, S. Paradis, P. H. Roy, M. Ouellette, and M. G. Bergeron.** 2001. Development of a PCR assay for identification of staphylococci at genus and species levels. J. Clin. Microbiol. **39:**2541-7.

128. **McDonald, C. P., A. Roy, P. Mahajan, R. Smith, A. Charlett, and J. A. Barbara.** 2004. Relative values of the interventions of diversion and improved donor-arm disinfection to reduce the bacterial risk from blood transfusion. Vox. Sang. **86:**178-82.

129. **McLaughlin, R. W., H. Vali, P. C. Lau, R. G. Palfree, A. De Ciccio, M. Sirois, D. Ahmad, R. Villemur, M. Desrosiers, and E. C. Chan.** 2002. Are there naturally occurring pleomorphic bacteria in the blood of healthy humans? J. Clin. Microbiol. **40:**4771-5.

130. **Melchior, W. B., Jr., and P. H. Von Hippel.** 1973. Alteration of the relative stability of dA-dT and dG-dC base pairs in DNA. Proc. Natl. Acad. Sci. U S A. **70:**298-302.

131. **Menitove, J. E.** 1990. Current risk of transfusion-associated human immunodeficiency virus infection. Arch. Pathol. Lab. Med. **114:**330-4.

132. **Menitove, J. E.** 1996. Transfusion-transmitted infections: update. Semin Hematol. **33:**290-301.

133. **Mitchell, K. M., and M. E. Brecher.** 1999. Approaches to the detection of bacterial contamination in cellular blood products. Transfus. Med. Rev. **13:**132-44.

134. **MMWR Morb Mortal Wkly Rep.** 1997. Red blood cell transfusions contaminated with *Yersinia enterocolitica*--United States, 1991-1996, and initiation of a national study to detect bacteria-associated transfusion reactions. MMWR. Morb. Mortal. Wkly. Rep. **46:**553-5.

135. **MMWR Morb Mortal Wkly Rep.** 1991. Update: *Yersinia enterocolitica* bacteremia and endotoxin shock associated with red blood cell transfusions--United States, 1991. MMWR. Morb. Mortal. Wkly. Rep. **40:**176-8.

136. **MMWR Morb Mortal Wkly Rep.** 1988. *Yersinia enterocolitica* bacteremia and endotoxin shock associated with red blood cell transfusion--United States, 1987-1988. MMWR. Morb. Mortal. Wkly. Rep. **37:**577-8.

137. **Morduchowicz, G., S. D. Pitlik, D. Huminer, M. Alkan, M. Drucker, J. B. Rosenfeld, and C. S. Block.** 1991. Transfusion reactions due to bacterial contamination of blood and blood products. Rev. Infect. Dis. **13:**307-14.

138. **Morrow, J. F., H. G. Braine, T. S. Kickler, P. M. Ness, J. D. Dick, and A. K. Fuller.** 1991. Septic reactions to platelet transfusions. A persistent problem. JAMA. **266:**555-8.

139. **Muder, R. R., Y. C. Yee, J. D. Rihs, and M. Bunker.** 1992. *Staphylococcus epidermidis* bacteremia from transfusion of contaminated platelets: application of bacterial DNA analysis. Transfusion. **32:**771-4.

140. **Muller-Alouf, H., C. Carnoy, M. Simonet, and J. E. Alouf.** 2001. Superantigen bacterial toxins: state of the art. Toxicon. **39:**1691-701.

141. **Myhre, B. A.** 1985. Bacterial contamination is still a hazard of blood transfusion. Arch. Pathol. Lab. Med. **109:**982-3.

142. **Myhre, B. A.** 1980. Fatalities from blood transfusion. JAMA. **244:**1333-5.

143. **Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter.** 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology. **148:**257-66.

144. **Nahlen, B. L., H. O. Lobel, S. E. Cannon, and C. C. Campbell.** 1991. Reassessment of blood donor selection criteria for United States travelers to malarious areas. Transfusion. **31:**798-804.

145. **Ness, P., H. Braine, K. King, C. Barrasso, T. Kickler, A. Fuller, and N. Blades.** 2001. Single-donor platelets reduce the risk of septic platelet transfusion reactions. Transfusion. **41:**857-61.

146. **Ness, P. M., and S. A. Campbell-Lee.** 2001. Single donor versus pooled random donor platelet concentrates. Curr. Opin. Hematol. **8:**392-6.

147. **Ness, P. M., and H. A. Perkins.** 1980. Transient bacteremia after dental procedures and other minor manipulations. Transfusion. **20:**82-5.

148. **Neubauer, H., L. D. Sprague, A. Hensel, S. Aleksic, and H. Meyer.** 2000. Specific detection of plasmid bearing *Yersinia* isolates by PCR. Clin. Lab. **46:**583-7.

149. **Olthius, H., C. Puylaart, C. Verhagen, and L. Valk.** 1995. Proc V Regional ISBT Congress.

150. **Ootsubo, M., T. Shimizu, R. Tanaka, T. Sawabe, K. Tajima, M. Yoshimizu, Y. Ezura, T. Ezaki, and H. Oyaizu.** 2002. Oligonucleotide probe for detecting Enterobacteriaceae by in situ hybridization. J. Appl. Microbiol. **93:**60-8.

151. **Opal, S. M., and J. Cohen.** 1999. Clinical gram-positive sepsis: does it fundamentally differ from gram-negative bacterial sepsis? Crit. Care. Med. **27:**1608-16.

152. **Panaccio, M., and A. Lew.** 1991. PCR based diagnosis in the presence of 8% (v/v) blood. Nucleic. Acids. Res. **19:**1151.

153. **Petershofen, E. K., R. Fislage, R. Faber, H. Schmidt, W. K. Roth, and E. Seifried.** 2000. Detection of nucleic acid sequences from bacterial species with molecular genetic methods. Transfus. Sci. **23:**21-7.

154. **Peterson, L. J., and R. Peacock.** 1976. The incidence of bacteremia in pediatric patients following tooth extraction. Circulation. **53:**676-9.

155. **Petrik, J.** 2001. Microarray technology: the future of blood testing? Vox. Sang. **80:**1-11.

156. **Pickard, C., L. Herschel, and P. Seery.** 1998. Visual identification of bacterially contaminated red blood cells. Transfusion. **38:**12S.

157. **Pitman, M.** 1953. A study of bacteria implicated in transfusion reactions and bacteria isolated from blood products. J. Lab. Clin. Med. **42:**273-288.

158. **Poljak, M., and K. Seme.** 1996. Rapid detection and typing of human papillomaviruses by consensus polymerase chain reaction and enzyme-linked immunosorbent assay. J. Virol. Methods. **56:**231-8.

159. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31:**775-82.

160. **Punsalang, A., J. M. Heal, and P. J. Murphy.** 1989. Growth of gram-positive and gram-negative bacteria in platelet concentrates. Transfusion. **29:**596-9.

161. **Quirk, M.** 2002. First VRSA isolate identified in USA. Lancet. Infect. Dis. **2:**510.

162. **Radstrom, P., A. Backman, N. Qian, P. Kragsbjerg, C. Pahlson, and P. Olcen.** 1994. Detection of bacterial DNA in cerebrospinal fluid by an assay for simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and streptococci using a seminested PCR strategy. J. Clin. Microbiol. **32:**2738-44.

163. **Rand, K. H., and H. Houck.** 1990. *Taq* polymerase contains bacterial DNA of unknown origin. Mol. Cell. Probes. **4:**445-50.

164. **Reading, F. C., and M. E. Brecher.** 2001. Transfusion-related bacterial sepsis. Curr. Opin. Hematol. **8:**380-6.

165. **Reik, H., and S. J. Rubin.** 1981. Evaluation of the buffy-coat smear for rapid detection of bacteremia. JAMA. **245:**357-9.

166. **Relman, D. A., and S. Falkow.** 1992. Identification of uncultured microorganisms: expanding the spectrum of characterized microbial pathogens. Infect. Agents. Dis. **1:**245-53.

167. **Relman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tompkins.** 1990. The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. N. Engl. J. Med. **323:**1573-80.

168. **Relman, D. A., T. M. Schmidt, R. P. MacDermott, and S. Falkow.** 1992. Identification of the uncultured bacillus of Whipple's disease. N. Engl. J. Med. **327:**293-301.

169. **Rhame, F. S., R. K. Root, J. D. MacLowry, T. A. Dadisman, and J. V. Bennett.** 1973. *Salmonella* septicemia from platelet transfusions. Study of an outbreak traced to a hematogenous carrier of *Salmonella* cholerae-suis. Ann. Intern. Med. **78:**633-41.

170. **Rider, J., and A. Newton.** 2002. Electrochemiluminescent detection of bacteria in blood components. Transfus. Med. **12:**115-23.

171. **Riley, H. D., Jr.** 1992. Changing patterns of infectious diseases. J. Tenn. Med. Assoc. **85:**423-5.

172. **Robins-Browne, R. M., S. Cianciosi, A. M. Bordun, and G. Wauters.** 1991. Pathogenicity of *Yersinia kristensenii* for mice. Infect. Immun. **59:**162-7.

173. **Robins-Browne, R. M., and J. K. Prpic.** 1985. Effects of iron and desferrioxamine on infections with *Yersinia enterocolitica*. Infect. Immun. **47:**774-9.

174. **Rock, G., D. Neurath, B. Toye, D. Sutton, A. Giulivi, J. Bormanis, B. Olberg, S. Holme, B. Wenz, G. Ortolano, and E. Nelson.** 2004. The use of a bacteria detection system to evaluate bacterial contamination in PLT concentrates. Transfusion. **44:**337-42.

175. **Roth, V. R., M. J. Kuehnert, N. R. Haley, K. R. Gregory, G. B. Schreiber, M. J. Arduino, S. C. Holt, L. A. Carson, K. V. Elder, and W. R. Jarvis.** 2001. Evaluation of a reporting system for bacterial contamination of blood components in the United States. Transfusion. **41:**1486-92.

176. **Rothman, R. E., M. D. Majmudar, G. D. Kelen, G. Madico, C. A. Gaydos, T. Walker, and T. C. Quinn.** 2002. Detection of bacteremia in emergency department patients at risk for infective endocarditis using universal 16S rRNA primers in a decontaminated polymerase chain reaction assay. J. Infect. Dis. **186:**1677-81.

177. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. **239:**487-91.

178. **Saiki, R. K., P. S. Walsh, C. H. Levenson, and H. A. Erlich.** 1989. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc. Natl. Acad. Sci. U S A. **86:**6230-4.

179. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning : a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

180. **Sarkar, G., and S. Sommer.** 1990. More light on PCR contamination. Nature. **347:**340-1.

181. **Sazama, K.** 1994. Bacteria in blood for transfusion. A review. Arch. Pathol. Lab. Med. **118:**350-65.

182. **Sazama, K.** 1990. Reports of 355 transfusion-associated deaths: 1976 through 1985. Transfusion. **30:**583-90.

183. **Schelstraete, B., B. Bijnens, and G. Wuyts.** 2000. Prevalence of bacteria in leucodepleted pooled platelet concentrates and apheresis platelets: current status in the Flemish Blood Service. Vox. Sang. **78:**P370.

184. **Scheu, P., A. Gasch, and K. Berghof.** 1999. Rapid detection of *Listeria monocytogenes* by PCR-ELISA. Lett. Appl. Microbiol. **29:**416-20.

185. **Sharma, J. K., V. Gopalkrishna, and B. C. Das.** 1992. A simple method for elimination of unspecific amplifications in polymerase chain reaction. Nucleic. Acids. Res. **20:**6117-8.

186. **Shoffner, M. A., J. Cheng, G. E. Hvichia, L. J. Kricka, and P. Wilding.** 1996. Chip PCR. I. Surface passivation of microfabricated silicon-glass chips for PCR. Nucleic. Acids Res. **24:**375-9.

187. **Shrestha, N. K., M. J. Tuohy, G. S. Hall, C. M. Isada, and G. W. Procop.** 2002. Rapid identification of *Staphylococcus aureus* and the *mecA* gene from BacT/ALERT blood culture bottles by using the LightCycler system. J. Clin. Microbiol. **40:**2659-61.

188. **Sleigh, J. W., and R. T. Cursons.** 2000. Generic polymerase chain reaction followed by DNA sequencing as a means of diagnosing bacteraemia. Anaesth. Intensive. Care. **28:**54-7.

189. **Soeterboek, A. M., F. H. Welle, J. H. Marcelis, A. Jansz, and C. M. van der Loop.** 1995. Prevalence of bacterial contamination in whole blood after donation. Vox. Sang. **69:**149.

190. **Sonnenwirth, A. C.** 1970. Bacteremia with and without meningitis due to *Yersinia enterocolitica*, *Edwardsiella tarda*, *Comamonas terrigena*, and *Pseudomonas maltophilia*. Ann. N Y Acad. Sci. **174:**488-502.

191. **Starr, D. P.** 1998. Blood : an epic history of medicine and commerce, 1st ed. Alfred A. Knopf, New York.

192. **Stenhouse, M. A., and L. V. Milner.** 1982. *Yersinia enterocolitica*. A hazard in blood transfusion. Transfusion. **22:**396-8.

193. **Suzuki, M., T. Mikami, T. Matsumoto, and S. Suzuki.** 1977. Gelation of *Limulus* lysate by synthetic dextran derivatives. *Microbiol Immunol.* **21:**419-25.

194. **Thisted Lambertz, S., A. Ballagi-Pordany, A. Nilsson, P. Norberg, and M. L. Danielsson-Tham.** 1996. A comparison between a PCR method and a conventional culture method for detecting pathogenic *Yersinia enterocolitica* in food. J. Appl. Bacteriol. **81:**303-8.

195. **Tipple, M. A., L. A. Bland, J. J. Murphy, M. J. Arduino, A. L. Panlilio, J. J. Farmer, 3rd, M. A. Tourault, C. R. Macpherson, J. E. Menitove, and A. J. Grindon.** 1990. Sepsis associated with transfusion of red cells contaminated with *Yersinia enterocolitica*. Transfusion. **30:**207-13.

196. **Trebesius, K., D. Harmsen, A. Rakin, J. Schmelz, and J. Heesemann.** 1998. Development of rRNA-targeted PCR and in situ hybridization with fluorescently labelled oligonucleotides for detection of *Yersinia* species. J. Clin. Microbiol. **36:**2557-64.

197. **Trebesius, K., L. Leitritz, K. Adler, S. Schubert, I. B. Autenrieth, J. Heesemann, and V. A. Kempf.** 2000. Culture independent and rapid identification of bacterial pathogens in necrotising fasciitis and streptococcal toxic shock syndrome by fluorescence in situ hybridisation. Rapid identification of pathogens in blood. Med. Microbiol. Immunol (Berl). **188:**169-75.

198. **VanderBorght, A., A. van der Aa, P. Geusens, C. Vandevyver, J. Raus, and P. Stinissen.** 1999. Identification of overrepresented T cell receptor genes in blood and tissue biopsies by PCR-ELISA. J. Immunol. Methods. **223:**47-61.

199. **Wagner, S. J.** 1996. The potential for bacterial testing of blood products. Zentralbl. Bakteriol. **283:**253-7.

200. **Wagner, S. J., L. I. Friedman, and R. Y. Dodd.** 1994. Transfusion-associated bacterial sepsis. Clin. Microbiol. Rev. **7:**290-302.

201. **Wagner, S. J., G. Moroff, A. J. Katz, and L. I. Friedman.** 1995. Comparison of bacteria growth in single and pooled platelet concentrates after deliberate inoculation and storage. Transfusion. **35:**298-302.

202. **Wagner, S. J., and D. Robinette.** 1998. Evaluation of an automated microbiologic blood culture device for detection of bacteria in platelet components. Transfusion. **38:**674-9.

203. **Wagner, S. J., and D. Robinette.** 1996. Evaluation of swirling, pH, and glucose tests for the detection of bacterial contamination in platelet concentrates. Transfusion. **36:**989-93.

204. **Wagner, S. J., D. Robinette, and R. Dodd.** 1993. Factors affecting *Yersinia enterocolitica* (serotype O:8) viability in deliberately inoculated blood. Transfusion. **33:**713-6.

205. **Wagner, S. J., D. Robinette, M. Nazario, and G. Moroff.** 1995. Bacteria levels in components prepared from deliberately inoculated whole blood held for 8 or 24 hours at 20 to 24 degrees C. Transfusion. **35:**911-6.

206. **Walther-Wenke, G., R. Doerner, T. Montag-Lessing, H. Lange, B. Baerbel, D. Walter, W. Martin, T. Heiner, and B. Walter.** 1999. Bacterial contamination of blood components: results of a two year survey in Germany Working Party "Microbiological Investigations in Transfusion Medicine" of the Advisory Board of the German Ministry of Health. Transfusion. **39:**34S.

207. **Wang, R. F., S. J. Kim, L. H. Robertson, and C. E. Cerniglia.** 2002. Development of a membrane-array method for the detection of human intestinal bacteria in fecal samples. Mol. Cell Probes. **16:**341-50.

208. **Wannet, W. J., M. Reessink, H. A. Brunings, and H. M. Maas.** 2001. Detection of pathogenic *Yersinia enterocolitica* by a rapid and sensitive duplex PCR assay. J. Clin. Microbiol. **39:**4483-6.

209. **Ward, P. A.** 2004. The dark side of C5a in sepsis. Nat. Rev. Immunol. **4:**133-42.

210. **Warwick, S., M. Wilks, E. Hennessy, J. Powell-Tuck, M. Small, J. Sharp, and M. R. Millar.** 2004. Use of quantitative 16S ribosomal DNA detection for diagnosis of central vascular catheter-associated bacterial infection. J. Clin. Microbiol. **42:**1402-8.

211. **Wauters, G., K. Kandolo, and M. Janssens.** 1987. Revised biogrouping scheme of *Yersinia enterocolitica*. Contrib. Microbiol. Immunol. **9:**14-21.

212. **Webb, I. J., F. S. Coral, J. W. Andersen, A. D. Elias, R. W. Finberg, L. M. Nadler, J. Ritz, and K. C. Anderson.** 1996. Sources and sequelae of bacterial contamination of hematopoietic stem cell components: implications for the safety of hematotherapy and graft engineering. Transfusion. **36:**782-8.

213. **Wenzel, R. P., M. R. Pinsky, R. J. Ulevitch, and L. Young.** 1996. Current understanding of sepsis. Clin. Infect. Dis. **22:**407-12.

214. **Wheeler, A. P., and G. R. Bernard.** 1999. Treating patients with severe sepsis. N. Engl. J. Med. **340:**207-14.

215. **Wilkinson, T. J., B. M. Colls, T. Chambers, and R. B. Ikram.** 1991. Blood transfusion acquired *Yersinia enterocolitica* sepsis: two cases. N. Z. Med. J. **104:**120.

216. **Williamson, L., H. Cohen, E. Love, H. Jones, A. Todd, and K. Soldan.** 2000. The Serious Hazards of Transfusion (SHOT) initiative: the UK approach to haemovigilance. Vox. Sang. **78 Suppl 2:**291-5.

217. **Williamson, L. M., S. Lowe, E. M. Love, H. Cohen, K. Soldan, D. B. McClelland, P. Skacel, and J. A. Barbara.** 1999. Serious hazards of transfusion (SHOT) initiative: analysis of the first two annual reports. BMJ. **319:**16-9.

218. **Wilson, I. G.** 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. **63:**3741-51.

219. **Wilson, K. H., R. B. Blitchington, and R. C. Greene.** 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. **28:**1942-6.

220. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221-71.

221. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. U S A. **87:**4576-9.

222. **Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. Lawn.** 1985. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. U S A. **82:**1585-8.

223. **Yap, E. P., and J. O. McGee.** 1991. Slide PCR: DNA amplification from cell samples on microscopic glass slides. Nucleic. Acids. Res. **19:**4294.

224. **Yeates, C., M. R. Gillings, A. D. Davison, N. Altavilla, and D. A. Veal.** 1997. PCR amplification of crude microbial DNA extracted from soil. Lett. Appl. Microbiol. **25:**303-7.

225. **Yomtovian, R., H. M. Lazarus, L. T. Goodnough, N. V. Hirschler, A. M. Morrissey, and M. R. Jacobs.** 1993. A prospective microbiologic surveillance program to detect and prevent the transfusion of bacterially contaminated platelets. Transfusion. **33:**902-9.

226. **Zavizion, B., D. Serebryanik, I. Serebryanik, J. Chapman, and A. Purmal.** 2003. Prevention of *Yersinia enterocolitica*, *Pseudomonas fluorescens*, and *Pseudomonas putida* outgrowth in deliberately inoculated blood by a novel pathogen-reduction process. Transfusion. **43:**135-42.

227. **Zeni, F., B. Freeman, and C. Natanson.** 1997. Anti-inflammatory therapies to treat sepsis and septic shock: a reassessment. Crit. Care. Med. **25:**1095-100.

228. **Zerbini, M., G. Gallinella, E. Manaresi, M. Musiani, G. Gentilomi, and S. Venturoli.** 1999. Standardization of a PCR-ELISA in serum samples: diagnosis of active parvovirus B19 infection. J. Med. Virol. **59:**239-44.

229. **Zhou, H., J. G. Hickford, and K. F. Armstrong.** 2001. Rapid and accurate typing of *Dichelobacter nodosus* using PCR amplification and reverse dot-blot hybridisation. Vet. Microbiol. **80:**149-62.